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Creating a new allohexaploid *Brassica* crop: determination of fertility and stability in novel *Brassica* hybrids

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Abstract

The *Brassica* genus is an economically important group of diploid (single genome) and allotetraploid (two-genome) species used as oilseeds, vegetables and condiments. Although no allohexaploid (three-genome) *Brassica* species exists in nature, the production of an allohexaploid *Brassica* crop could be beneficial for agriculture. However, production of a stable and fertile *Brassica* allohexaploid is currently hindered by challenges of infertility and unstable meiosis. To date, incremental progress has been made in creating an allohexaploid species using several different species combinations. Despite this, a gap in knowledge currently exists for genomic stability among *Brassica* allohexaploids, critical in the establishment of a successful species.

Firstly, I investigated fertility and meiotic stability in 100 plants from the cross *B. carinata* × *B. rapa* (A2 allohexaploid population) and 69 plants from the cross (*B. napus* × *B. carinata*) × *B. juncea* (H2 allohexaploid population). Estimated pollen viability, self-pollinated seed set, number of seeds on the main shoot, number of pods on the main shoot, seeds per 10 pods and plant height were measured for both the A2 and H2 populations and a set of reference control cultivars. The H2 population had high segregation for pollen viability and meiotic stability, while the A2 population was characterised by low pollen fertility and a high level of chromosome loss. Both populations were taller, but had lower average fertility trait values, than the control cultivar samples. Additionally, I established that the genotypes of the parents and H1 hybrids are affecting chromosome pairing and fertility phenotypes in the H2 population.

Next, I investigated fertility, meiosis and genetic variability in sets of self-pollinated progenies (the MDL2 population) resulting from first generation microspore-derived plants (the MDL1 population). These populations were derived from microspores of a near-allohexaploid interspecific hybrid plant from the cross *(Brassica napus* × *B. carinata*) × *B. juncea.* Fertility decreased from MDL1 to MDL2 population, with several fixed chromosome duplications and deletions present as well as novel genomic events seen to have occurred from the MDL1 to MDL2. Genetic non-identity between lines within various progeny sets in MDL2 was also observed, uncharacteristic of what would be expected of a microspore-derived (normally doubled haploid; DH) population.

Finally, I made novel interspecific hybrids between wild C genome species, *B. oleracea* and *B. juncea* to utilise this germplasm for the creation of a diverse allohexaploid species. Hand pollinations between two genotypes of *B. juncea* (Xingyou 4 and B578) and *B. oleracea* (TO1000) and wild C genome species *B. incana*, *B. montana* and *B. cretica* were performed (747 total bud pollinations, average 62.25 per cross combination) in both cross directions. The combination with *B. oleracea* produced six triploid hybrids (2n = ABC) from 85 flower pollinations. Pollen fertility in triploid hybrids was low; between 2 – 10% (average fertility 5.8%). Flow cytometry and phenotypic observations confirmed hybrids as 3*x* triploids; no pod or seed setting was observed in any of these hybrids. Confirmed (3*x*) hybrids were multiplied and treated with colchicine chemical treatment of varying concentrations to induce chromosome doubling. Colchicine-treated hybrids revealed changes in pods, leaves and stems relative to untreated controls. Seed development was observed in all treatments, with the 0.15% and 0.2% treatment groups producing the most seeds (62 and 58 seeds respectively). A total of 200 seeds were harvested from the S_0 generation plants, of which 140 seeds were sown and 94 plants germinated to give rise to the S_1 generation. A total of 94 plants from the S_1 generation germinated and showed an average of 57% pollen viability (range 7 – 84%). This material shows promise for integration of genetic material from *B. oleracea* and *B. juncea* into a new allohexaploid crop species.

From research carried out, I found ongoing segregation for fertility and meiotic stability in two novel allohexaploid *Brassica* populations. Also, I found that despite the distinct advantages in using microspore culture to develop doubled haploid populations for breeding or genetics studies, there is potential for meiotic instability to cause undesirable genetic non-identity. Finally, I outlined the creation of a new allohexaploid *Brassica* species from *B. oleracea* × *B. juncea* hybrids. This thesis and the various studies undertaken all share a common theme, i.e. the determination of fertility and genome stability in allohexaploid material. From this research, I have contributed to the development of new allohexaploid germplasm material resources. Additionally, I have contributed to the overall pool of knowledge about *Brassica* allohexaploids, providing beneficial information for researchers and breeders involved in studying and developing allohexaploid *Brassica* for agricultural benefit.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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Chapter 4

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Chapter 5

Dr. Aneeta Pradhan assisted with hybrid plants in glasshouse and the colchicine treatment experiment.

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allohexaploid, *Brassica*, chromosome inheritance, doubled haploids, fertility, SNP genotyping, meiosis

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List of Abbreviations

AGG	Australia Grains Genebank
ANOVA	Analysis of Variance
BAP	Benzyl Amino Purine
BSA	Bulk Segregant Analysis
CNV	Copy Number Variation
CV	Covariance
DAP	Day after pollination
DH	Double Haploid
DMSO	Dimethyl sulfoxide
DNA	Deoxy Nucleic Acid
FACs	Fluorescence activated cell sorting
FC	Flow Cytometry
FCM	Flow Cytometry
$g.l^{-1}$	gram per litre
GA_3	Gibberellic Acid
HSD	Highly Significant difference
IAA	Indole Acetic Acid
Mbp	Mega base pair
MDL	Microspore-derived line
Mg	milligram
$mg.l^{-1}$	milligram per litre
MS	Murashige and Skoog
Mya	Million years ago
NGS	Next Generation Sequencing
PAV	Presence Absence Variation
PGF	Plant Growth Facility
PI	Propidium Iodine
PMC	Pollen Mother Cell
QTL	Quantitative Trait Loci
SNP	Single Nucleotide Polymorphism
TBE	Tris Boric Acid EDTA
WGD	Whole Genome Duplication

WGT	Whole Genome Triplication

Chapter 1: General Introduction

1.1 Polyploidy and interspecific hybridisation

Polyploidy, also known as whole genome duplication (WGD), is an important occurrence among eukaryotes and is proposed to be a main source of evolutionary genomic variation and plasticity. Additionally, it can be described as the presence of more than two complete chromosome sets within an organism (Soltis et al. 2004; Comai 2005; Arrigo and Barker 2012; De Storme and Mason 2014; Soltis et al. 2015). There are different types of polyploids: autopolyploids are polyploids which contain more than two copies of the same genome, while allopolyploids have a combination of genomes from more than one ancestral species. Aneuploids are polyploids with an addition or subtraction of one or more specific chromosomes relative to the total set of chromosomes in a species' genome (Ramsey and Schemske 1998). Diploids (most somatic cells) consist of two sets of chromosomes from each parent ($2n = 2x$), also known as homologous pairs. Haploids have only one of the two parental chromosome sets ($n = x$), as found in gamete cells e.g. pollen and ovules (Ramsey and Schemske 1998; Soltis 2013).

Polyploidy has been described as an essential phenomenon in evolution and adaptation and plays a critical role in the formation of fertile species in crops (Liu et al. 2006; Marhold and Lihova´ 2006; Gaeta et al. 2007; Abbott et al. 2013; Soltis et al. 2014). Moreover, phylogenetic analysis of sequenced plant genomes identified ancient whole genome duplication events in the common ancestors of extant seed plants and angiosperms, indicating that all flowering plants have gone through polyploidization events (Jiao et al. 2011). Some researchers describe polyploidy to be an evolutionary "dead end", suggesting selection becomes inefficient when genes are present in multiple copies (Mayrose et al. 2011). Soltis et al. (2014), however, state that polyploid species can maintain high levels of segregating genetic variation through the incorporation of genetic diversity from their diploid progenitors and can generate novel genetic variation. Many economically important food crops are ancient or recent polyploids, usually resulting from unintentional hybridisation or selective breeding. Three *Brassica* diploid species *B. rapa*, *B. oleracea* and *B. nigra* combined naturally through interspecific hybridisation in a pairwise fashion to form three allotetraploid species i.e. *B. napus, B. carinata* and *B. juncea* (U 1935).

1.2 Mechanism of polyploid formation

Polyploidy arises in nature through different pathways. For instance, polyploidy may occur in somatic cells following a failure in cell division after mitosis in the zygotes or meristems (Bretagnolle and Thompson 1995). However, the success and stability of somatic whole genome replication and the transmission of this kind of polyploidy to subsequent generations is dependent on the method of reproduction and tissue type involved (De Storme and Mason 2014). Another pathway for polyploid formation is through gametic non-reduction, which commonly occurs via "meiotic nuclear restitution" where a failure in cell division during meiosis leads to the formation of unreduced (2n) gametes with the full somatic chromosome number. Harlan and de Wet (1975) state that unreduced gametes constitute a major mode of polyploid formation, whereas somatic doubling mechanisms have only a minor contribution. Mason and Pires (2015) propose polypoid formation pathways can be categorised into either one step, triploid hybrid, hybrid bridge or somatic doubling pathways. The one step pathway involves the direct union of two unreduced gametes, while triploid hybrids can play a role as triploid bridges: the union of a reduced and an unreduced gamete can lead to the development of triploid embryos which can backcross to a parent to make balanced tetraploids (Ramsey and Schemske 1998; Otto and Whitton 2000). Chromosome doubling may also be induced artificially by application of chemicals such as colchicine (used to arrest spindle fibre formation at meiosis) which is often used to restore fertility in F_1 hybrids (Marasek-Ciolakowska et al. 2016).

Somatic doubling is commonly done to produce synthetic polyploids (Tayalé and Parisod 2013), this can be through doubling a single diploid genome to create autotetraploids, or by doubling chromosome numbers in interspecific hybrids to create allotetraploids. Synthetic polyploids provide excellent genetic material for comparative analysis of gene expression and genomic changes during the initial stages of polyploid formation (Chen and Ni 2006). Abel et al. (2005) developed synthetic *Brassica napus* for analysis of fixed heterosis, while in Song et al. (1995) synthetic polyploids of *Brassica* were used to investigate polyploid evolution. Also, resynthesized *Brassica napus* were used to investigate meiosis, genetic changes and mechanisms involved in stabilizing these progeny (Szadkowski et al. 2010).

In the fruit industry, artificial breeding is applied in developing seedless watermelons, whereby seeds and seedlings of diploid watermelon are treated using colchicine, doubling the genome

to produce tetraploid watermelons (Song et al. 2012). Furthermore, in citrus breeding, interploid crosses are made between diploids to create triploid citrus plants. Other important fruits produced from triploids include varieties of apple, bananas, mulberry, sugar beets and tea (Jaskani et al. 2005).

1.3 The *Brassica* genus

Brassica species are important in agriculture and belong to the Brassicaceae (Cruciferae) family. They are a rich source of edible oil, vegetables, and protein and oil, and have potential as renewable raw materials for biofuel production (Rakow 2004; Branca and Cartea 2011; Chen et al. 2011). The triangle of U (Figure 1.1; U 1935) is an evolutionary example of allopolyploidy and illustrates the ancestral relationship that exists between the *Brassica* species. Historically, three progenitor diploid species; *B. rapa* (A genome, 2n = 20, Chinese cabbage and turnip), *B. nigra* (B genome, 2n = 16, black mustard) and *B. oleracea* (C genome, 2n = 18, cabbage, cauliflower, broccoli) shared their unique genomes through pairwise hybridisation and chromosome doubling. This event gave rise to three allotetraploid species; *B. juncea* (A and B genome, 2n = 36, leaf mustard, Indian mustard), *B. napus* (A and C genome, 2n = 38, oilseed rape, canola) and *B. carinata* (B and C genome, 2n = 34, Ethiopian mustard) (Morinaga 1934; U 1935).

The Brassicaceae contains evidence of three ancient whole genome duplication (WGD) events, the α, β and γ events (Franzke et al. 2011). The α event, which is the most recent, dates from approximately 47 Mya (Bowers et al. 2003). *Brassica* diploid species are also mesopolyploids, having undergone an hexaploidization event approximately 23 Mya (Parkin et al. 2014). This event was followed by massive gene loss and frequent reshuffling, a key factor in speciation and morphotype diversification (Liu et al. 2013; Cheng et al. 2014). The A and C genomes diverged more recently from a common ancestor, while the B genome is described as being more distantly related (Lagercrantz and Lydiate 1996).

Despite the fact that each pair of genomes in these *Brassica* species can co-exist in an allotetraploid species, a natural *Brassica* allohexaploid species (2n =AABBCC) does not exist (Mason et al. 2014). However, potential exists to develop a new allohexaploid crop for agriculture (Chen et al. 2011). As well, *Brassica* researchers and breeders have been able to

exploit the genetic resources within the triangle of U for crop improvement by using trigenomic bridges. Trigenomic bridges are *Brassica* interspecific hybrid plants containing the three *Brassica* genomes (A, B and C) in various combinations: this can either be triploid (ABC), unbalanced tetraploid (e.g. AABC), pentaploid (e.g., AABCC) or hexaploid (AABBCC) (Chen et al. 2011).

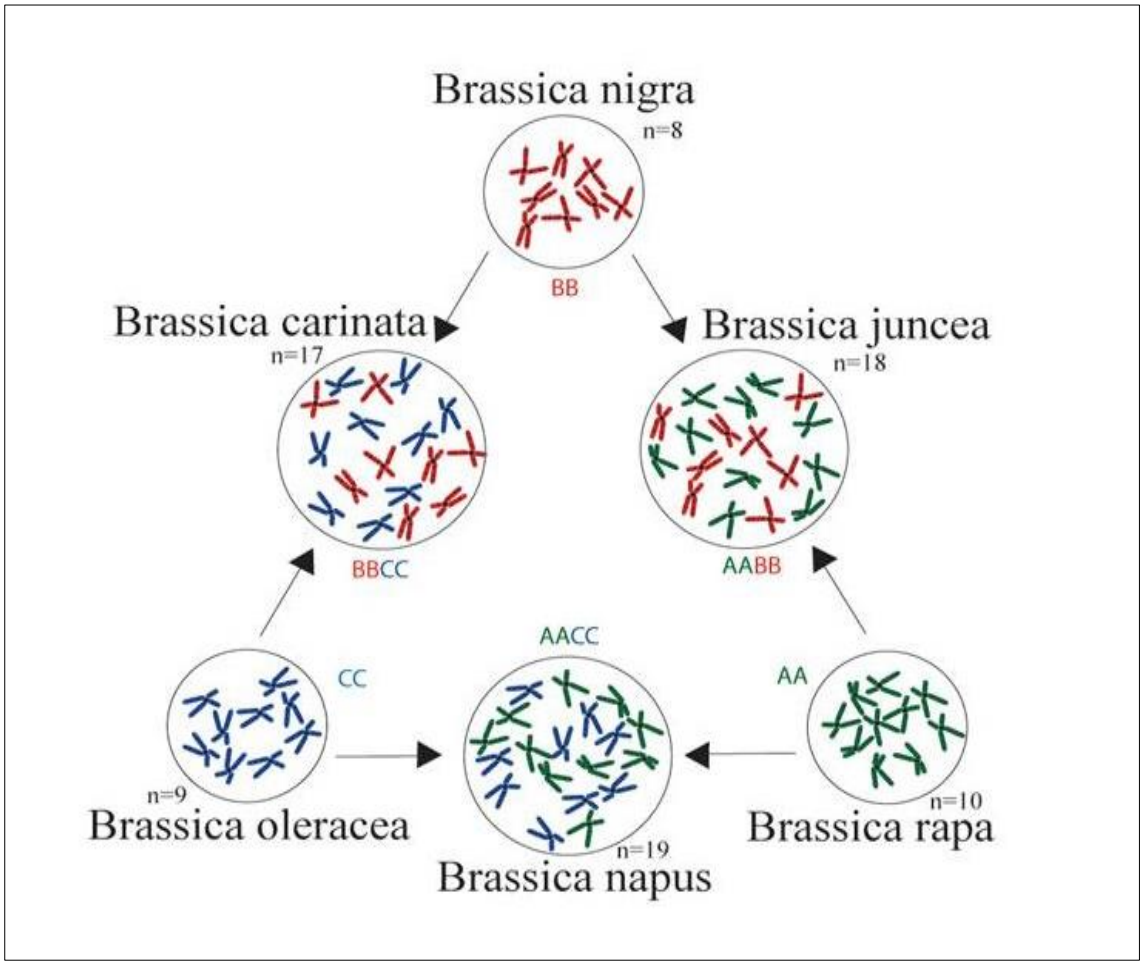

Figure 1.1 Relationship of the six natural *Brassica* species in the triangle of U (Adapted from U 1935).

1.4 The benefits of polyploidy and significance in agriculture

Genetic changes arising because of polyploid formation include deletions, translocations, homoeologous exchanges and epigenetic modification, while gene expression changes include genetic dominance, genetic silencing, novel activation and sub-functionalisation (Chen and Ni 2006; Gaeta et al. 2007). Benefits arising because of polyploidy include heterosis, which can cause polyploids to be more vigorous than their diploid progenitors, and gene redundancy, which can shield polyploids from deleterious effects of mutations (Comai 2005). The larger phenotypic and ecological ranges evident in new polyploids compared to their ancestors is a potentially significant feature for successful polyploid establishment and speciation (Arrigo and Barker 2012; Soltis et al. 2015). This variation has been viewed as a strategy to avoid competition with their established diploid parents and escape minority cytotype disadvantage (Abbott et al. 2013).

Other advantages occurring from polyploid formation include increased allelic diversity, genetic variation and heterozygosity, additionally polyploids are often more adaptable to a broader range of ecological conditions than their progenitors (Udall and Wendel 2006; Arrigo and Barker 2012). Bread wheat (*Triticum aestivum*) is an example of a hexaploid crop with A, B and D genomes. Bread wheat shows benefits accrued from polyploidization, such as wider adaptation than its lower ploidy relatives: it can be grown from the tropics to cold temperate agricultural regions (Leitch and Leitch 2008). Additionally, tetraploid cotton which has the A and D genomes is preferred in fibre production compared to its diploid progenitors due to the improved quality in its longer, stronger, finer fabric and higher yields (Udall and Wendel 2006; Chee et al. 2016).

Difficulties associated with polyploid formation include changes in cellular architecture and regulatory implications, as well as aneuploidy arising from abnormal meiosis and epigenetic instability (Soltis et al. 2004; Comai 2005). In artificial synthetic polyploid *Brassica* hybrids, extensive genomic rearrangements, point mutations, DNA methylation and fragment losses were observed (Song et al. 1995). While in Xiong et al. (2011), initial generations of resynthesized *B. napus* were shown to involve aneuploidy and gross chromosomal rearrangements, while dosage balance mechanisms enforced chromosome number stability. Meanwhile, Szadkowski et al. (2010) found massive genetic changes in resynthesized *Brassica napus* which were carried over to the progenies.

Plant breeders can exploit the advantages of polyploidy in their breeding programs by artificial induction of polyploids using techniques of distant hybridisation and tissue culture, protoplast culture and somatic hybridisation followed by selective breeding. Applications may include the production of high yielding fruit trees, horticultural plants and vegetables, as well as ornamental and medicinal plants (Ramsey and Schemske 1998; Song et al. 2012).

1.5 Interspecific hybridisation for crop improvement in *Brassica*

Breeders have over the years used interspecific hybridisation to increase genetic variability within species, and to improve cultivated species to withstand abiotic and biotic stresses for successful crop production. Related or wild species in a genus contain a large reservoir of genes covering a variety of desirable traits, thus by tapping into, and utilising this germplasm, there is great potential for agricultural crop improvement (Liu et al. 2005). A number of studies have been carried out in the past investigating interspecific hybridisation and the relationship between species in *Brassica.* (FitzJohn et al. 2007). The interrelationship between the diploid and tetraploid *Brassica* species, as evident in U's Triangle (Morinaga 1934; U 1935), is an example of how natural hybridisation events can form a basis for genome evolution. This relationship also demonstrates how interspecific crosses enable gene exchange and contribute to the differentiation of a genus by generating new types of hybrids across species boundaries (Branca and Cartea 2011).

Trait transfer among economically important *Brassica* species and genera can be achieved either by hand pollination, unassisted pollination, somatic hybridisation or embryo rescue techniques (FitzJohn et al. 2007). The presence of reproductive barriers, however, remains a major limitation, further restricting gene transfer to mainly sexually-compatible species. However, advances in *in-vitro* culture and embryo rescue have shown an ability to overcome these barriers, enabling transfer of traits among previously incompatible species and genera (Waara and Glimelius 1995).

Wild species are an important source of genes for improvement of cultivated crops, the relationship and potential to make crosses between wild and cultivated species has been studied at length (Inomata 1993b; Lannér et al. 1997). Interspecific hybridisation between plant species and wild relatives has often been used to transfer useful traits, such as disease resistance, from wild to cultivated species. *Brassica maurorum* (M genome, resistant to Alternaria blight and

white rust) is a wild species that has been successfully crossed with all six-cultivated species of *Brassica* through embryo rescue (Chrungu et al. 1999). Other studies replicated crosses to characterize inter- and intra-genomic relationships and relatedness of the M genome to the A/B/C genomes (Yao et al. 2010, 2012). Also, Inomata (1993a) examined crossability and cytology in hybrids and F_1 plants from *B. campestris* and *B. montana*, *B. cretica* and *B. bourgeaui* hybrids. The F_1 plants obtained were crossed with *B. napus* to examine the potential of transferring important agronomical traits to cultivated *Brassica.* Additionally, in a study by Bhaskar et al. (2002), hybrids were made between a wild species, *Erucastrum canariense,* and a cultivated *B. rapa* to examine their potential to transfer resistance to Alternaria blight and mustard aphid.

1.6 Allohexaploid *Brassica*

The production of an allohexaploid *Brassica* to harness benefits of polyploidisation has been a topic of continuing interest to *Brassica* breeders and researchers. An allohexaploid *Brassica* species could be developed for tolerance to a range of environmental conditions, based on the observation that many allopolyploid species are more widely adaptable compared to their lower ploidy parent species (Chen et al. 2011). Most polyploids behave as diploids in meiosis, indicating that the precise control of pairing at meiosis is important and confers evolutionary advantages in polyploid species (Jenczewski et al. 2003). Presently, no known stable *Brassica* allohexaploid exists in naturally in agriculture (Chen et al. 2011; Mason and Batley 2015). Some early studies and attempts to create *Brassica* allohexaploids involved induction of somatic chromosome doubling of triploid ABC interspecific hybrids to form AABBCC allohexaploids (Iwasa 1964). Howard (1942) produced 2n = AABBCC plants from the cross *B. rapa* and *B. carinata* that restored high fertility over a few generations. Various studies have also been conducted to use allohexaploid *Brassica* as a bridge to transfer useful traits such as disease resistance between species (Sjödin and Glimelius 1989; Arumugam et al. 1996; Meng et al. 1998; Rahman 2001; Li et al. 2004). Recently, Gupta et al. (2016) reported for the first time development of a stable allohexaploid species between *B. rapa* and *B. carinata,* with stability confirmed in two allohexaploid combinations up to the H_4 generation in two different locations in India.

The most common strategy to produce trigenomic triploids is to make crosses between the tetraploid and diploid species to produce ABC hybrids, followed by chromosome doubling using the spindle-fibre-inhibiting agent colchicine (Chen et al. 2011). Although colchicine is generally successful at inducing chromosome doubling in most genera, alternative chemicals that can be used to induce *in-vitro* chromosome doubling include trifluralin and oryzalin (Hansen and Andersen 1996). In a study to create interspecific hybrids from crosses between *B. napus* × *B. nigra,* putative hybrid seeds were confirmed using cytological analysis to contain 27 chromosomes (Pradhan et al. 2010); these triploids were subsequently doubled to produce allohexaploids.

Other studies on allohexaploids synthesised from *B. carinata* × *B. rapa* have shown an increase in meiotic stability with each progressive generation, while the proportion of progeny with 54 chromosomes increased with successive self-pollination and selection in each generation (Tian et al. 2010). In Mason et al. (2014), near-allohexaploid *Brassica* hybrid populations with variable fertility and chromosome inheritance were produced from the species combination (*B. napus* × *B. carinata*) × *B. juncea*). Meanwhile, Zhou et al. (2016) synthesised *Brassica* allohexaploids that were created using three different crossing strategies, and investigation was carried out over several generations. In this study euploid and aneuploid progenies were produced and were used to investigate genomic stability, which was found to follow the B > A > C pattern. Also, the entire loss of the C genome was found to lead to development of progeny resembling *B. juncea*.

The formation of unreduced (2n) gametes, described as an important mechanism in polyploid formation, has been observed in a numerous plant including *Brassica* (Nelson et al. 2009). There are different pathways that result in the formation of viable unreduced gametes, they include meiotic abnormalities such as omission of the first or second meiotic division, abnormal spindle morphology in the second division, and disturbed cytokinesis. Additionally, three genetically different types of 2n gametes can be identified based on how the nucleus restitutes: first division restitution (FDR), second division restitution (SDR), or indeterminate meiotic restitution (IMR) (Marasek-Ciolakowska et al. 2016). In some cases, unreduced gametes may also lack a complete euploid chromosome complement as a result of additional disturbances to chromosome pairing and segregation, resulting in gametes which although unreduced may also be aneuploid or contain non-homologous chromosome rearrangements

(Nelson et al. 2009; Mason et al. 2011, 2012). Mason et al. (2012) designed a two-step crossing strategy using unreduced gametes to produce trigenomic allohexaploid *Brassica* lines. Firstly, a pairwise crossing of the three allotetraploid *Brassica* species described in the U Triangle is carried out, leading to production of unbalanced trigenomic hybrids. Subsequently, these are the crossed in the second step with complementary reduced gametes to produce putative AABBCC allohexaploids

1.7 Hybridisation barriers

One of the main obstacles in *Brassic*a breeding programmes is a narrow genetic variability. However, one strategy to overcome this is to use wild and weedy relatives of *Brassica* crop species, which are a rich reservoir of genes conferring resistance/tolerance to biotic and abiotic stresses. Wide hybridisation between wild and cultivated species is a critical approach that is used to capture desirable traits. However, the presence of sexual barriers can prevent successful interspecific hybridisation: these can be either pre- or post-fertilisation barriers (Inomata 1993b; Chrungu et al.1999).

High levels of incompatibility in many interspecific crosses may occur because of a lack of genetic information from one partner, preventing successful completion of pollination processes in the other (Inomata 1993b). Pollination incompatibility occurs when pollen-pistil interactions prevent the formation of a viable zygote. Several *in-vitro* methods can be used to overcome incongruity barriers in plant species. Pre-fertilization barriers can be overcome using i*n-vitro* pollination and fertilization while post-fertilization barriers may be overcome by the culture of ovaries immediately after pollination, or by ovule culture. A combined approach i.e. *in-vitro* pollination and fertilization are commonly used for some plant groups (Tuyl et al. 1991; Tuyl and Jeu 1997). The utilization of *in-vitro* methods and embryo rescue can enable utilization of genetic reservoir among wild *Brassica* species (Zhang et al. 2003). Mohapatra and Bajaj (1987) describe production of interspecific hybrids between an incompatible combination of *Brassica juncea* × *Brassica hirta* through *in-vitro* culture of ovules and ovaries.

Embryo rescue is a useful technique applicable in interspecific hybrids in instances when abortion of hybrid embryos is likely to happen after fertilization (Tu et al. 2008; Branca and Cartea 2011). This strategy has been used in *Brassica* where interspecific and intergeneric

hybrids are produced by excising embryos from the ovaries or ovules. However, in cases where this is not possible, whole ovules or even ovaries containing them are cultured (Inomata 1993b; Sharma et al. 1996). Embryo culture techniques are useful for improving the efficiency of interspecific crosses, obtaining plants from inherently weak embryos, and for regenerating haploid plants for a shortened breeding cycle (Zhang et al. 2003).

1.8 Fertility and meiotic stability barriers

In plant sexual reproduction, viable pollen is required to deliver male gametes to the embryo for successful fertilisation. Pollen quality is used to assess plant fertility and fertilisation, which is vital in agriculture since pollen must be viable at pollination for seed set to occur (Heslop-Harrison 1992). Meiosis leads to the formation of sexual gametes in species and contributes to genome stability and diversity, thus a correct segregation process at metaphase leads to the production of stable and viable gametes while partial fertility and aneuploidy (characterised by poor/no seed set) often happen if abnormal meiosis occurs (Cifeuntes et al. 2010b).

The establishment of a new polyploid species requires control of chromosome pairing involved in meiosis to produce balanced gametes and to assure genome stability in future generations (Jenczewski and Alix 2004; Gaeta and Pires 2010; Grandont et al. 2014). New species arising from interspecific hybrids often do not have stable meiosis in the earlier generations, resulting in non-homologous interactions between chromosomes from different sub genomes during meiosis, and leading to chromosome loss, instability and infertility (Mason and Batley 2015). Formation of bivalents is necessary for stable meiosis to occur because intergenomic recombination compromises the maintenance of two chromosome complements (Comai 2005). The mechanisms through which meiosis in polyploids return to a “diploid-like” chromosome segregation pattern without associations between sub genomes is known as cytological diploidization (Cifuentes et al. 2010b).

In allotetraploid oilseed rape (*Brassica napus*) several quantitative trait loci (QTL) with effects on meiotic behaviour have been identified, including the pairing regulator in *B. napu*s (*PrBn*) locus, which has been shown to have quantitative effects on crossover frequency (Jenczewski et al. 2003; Liu et al. 2006; Nicolas et al. 2009). Mason and Batley (2015) suggest that genetic control of meiosis in *B. napus* may have resulted from novel genetic variation in the newly

formed allotetraploid, or by the accumulation of minor alleles inherited from the parent diploids. The study of genetic factors controlling meiosis in bread wheat suggests that meiotic interactions between homeologues are genetically controlled by the *Ph1* locus, which has now been characterised at a molecular level (Griffiths et al. 2006). The three distinct, yet related, genomes coordinate meiotic pairing, such that all three sets of chromosomes (A, B and D genomes) pair faithfully with their homologs and segregate disomically, while mutations at this locus lead to homoeologous recombination and gross chromosomal rearrangements (Gaeta and Pires 2010).

A study by Tian et al. (2010) to synthesise *Brassica* allohexaploids between *B. carinata* and *B. rapa* found the A and B genomes to be less stable when compared to the C genome, possibly occurring due to rearrangements between the A and C genomes. In allohexaploid *Brassica*, Geng et al. (2013) observed abnormal chromosome behaviour during meiosis in hexaploid *Brassica* parents and doubled-haploid progeny. Mason et al. (2014) report low frequencies of homoeologous recombination across most of the genomes in a study investigating fertility, chromosome transmission and genetic stability in self-pollinated progeny of near-allohexaploids. Meanwhile, Gupta et al. (2016) synthesised a stable allohexaploid between *B. rapa* and *B. carinata* and found that complete chromosome complements of all three genomes were retained with no major chromosome loss or translocations. Additionally, Mwathi et al. (2017) reported decreased fertility and chromosome loss in an allohexaploid population from *B. carinata* × *B. rapa* crosses coupled with variable fertility in second generation allohexaploids from the cross (*B. napus* × *B. carinata*) × *B. juncea*. However, little is known about the genetic and genomic factors influencing meiotic behaviour and genomic stability in *Brassica* allohexaploids.

1.9 Doubled haploid (DH) technology

Haploid tissue cultures are most often obtained by culturing microspores, pollen grains, and anthers. Their use allows the possibility of isolating an array of individual genomes whether dominant or recessive, providing immediate benefit for breeding and genetic analysis (Cousin et al. 2009; Altman and Hasegawa 2012). Haploid plants produced from an "F_1" combine the two parental genomes but have only one allele at every locus. Converting these sterile haploids into fertile diploids produces immortal homozygous lines (Seymour et al. 2012).

DH mapping populations have been made in important agricultural crops such as rice, maize, and *Brassica* oilseeds (Xu et al. 2007; Seymour et al. 2012). Among *Brassica* species microspore culture is routinely used to produce homozygous doubled haploid lines for breeding and experimental purposes and is also important for genetic and genomic studies (Geng et al. 2013). One of the benefits of using microspore-derived (MD) progeny to understand meiosis in hybrids is the ease involved in genotyping because of the genetic material originating from a single gamete (Nelson et al. 2009). In Mason et al. (2015), double haploids were produced and used to further investigate meiotic behaviour in *Brassica* allohexaploids. In Geng et al. (2013), double haploid mapping populations were also developed from hexaploid *Brassica* from different sources for further genetic studies.

1.10 Genomic analysis and high-throughput genotyping for crop improvement

Advances in genomics technologies have led a better understanding of crop genomes and an improvement in agricultural productivity. This is possible through the discovery of genetic variation important in increasing performance and efficiency of plant breeding (Edwards et al. 2013; Bevan et al. 2017). For instance, reference genomes of various important food crops and an increasing number of wild relatives are now available. Also, efforts are ongoing to capture genetic diversity of African orphan crops through various initiatives (Morrell et al. 2011; Batley and Edwards 2016). Sequencing techniques have rapidly developed from the traditional Sanger sequencing chain termination method to the current second or next-generation sequencing (NGS), an important tool in plant breeding (Mason and Batley 2015).

Different market leaders continue to dominate this dynamic field of agriculture genomics providing different platforms, each offering different costs per sample, read lengths, run times and accuracies (Quail et al. 2012; Goodwin et al. 2016). Second generation sequencing describes platforms that produce large amounts (usually millions) of short DNA sequence reads from 25 bp to the current portable Oxford nanopore MinION with reads exceeding 150 kb (Edwards and Batley 2010; Jain et al. 2016). Next generation sequencing provides an enormous number of reads through massive parallelisation and in the process thus permitting sequencing of entire genomes at a fraction of the costs for Sanger sequencing (Futschik and Schlotterer 2010). However, with the emergence of these new technologies, new challenges are created,

mainly through the production of vast quantities of data that require careful analysis (Goodwin et al. 2016). The complete assembly of a genome sequence makes it simpler to do re-sequencing of other individuals of the same species: this is because sequence reads can easily be mapped to the assembled reference genome (Hayward et al. 2012). The genomes of *B. napus* (Chalhoub et al. 2014), *B. oleracea* (Liu et al. 2014; Parkin et al. 2014), *B. rapa* (Wang et al. 2011) and *B. juncea* (Yang et al. 2016) have now been completely sequenced. The availability of these genomic resources coupled with advances in next generation sequencing can be simultaneously used to probe genomic diversity and chromosome evolution, potentially providing the clues to discovery of genetic mechanisms influencing homeologous chromosome recombination.

The most commonly applied molecular tools for crop improvement are molecular genetic markers. Single nucleotide polymorphisms (SNPs) are genetic markers based on single nucleotide substitutions of one base for another in DNA sequences, they are categorised as transversions, transitions, and insertions/deletions. SNPs remain the preferred choice for numerous research and breeding applications because of their high prevalence in the genome and potential for strong linkage to selected traits (Hayward et al. 2012; Huang et al. 2013; Dalton-Morgan et al. 2014).

High-density SNP genotyping arrays are a powerful tool for studying genomic patterns of diversity, inferring ancestral relationships between individuals in populations, and investigating marker–trait associations in mapping experiments (Wang et al. 2014). The use of SNP arrays coupled with the now readily-available reference genomes has become a favourite genomic tool with *Brassica* researchers, mainly due to the speed of acquisition of the high-throughput data as well as ease of the analysis involved. SNP arrays have been used across a wide range of studies including molecular karyotyping, germplasm collection characterization as well as for genotyping yield, vigour and phenotype related characteristics. (Clarke et al. 2016; Mason et al. 2017). Until recently only the *Brassica* Illumina Infinium 60K A/C SNP array was available. A new 90K A/B/C SNP chip, with SNPs distributed across the A, B and C genomes is now currently available, providing the ability genotype trigenomic *Brassica* experimental populations containing all three genomes.

Genomic rearrangements occurring because of homoeology between the A and C genomes are a common occurrence in interspecific hybrids, where they can become an important source of genetic and phenotypic variation (Nicolas et al. 2007, 2012; Stein et al. 2017). Meiosis events in *B. napus* leads to crossovers between A and C chromosomes resulting in the production of gametes with balanced (reciprocal) and unbalanced (non-reciprocal, duplication or deletion) translocation events. Though self-pollination, duplications and deletions are fixed in populations, resulting in both copy number variation (CNV) and presence absence variation (PAV) which are visualized through SNP arrays (Mason et al. 2017). Presently, however, little is known about the impact of CNVs at the cellular and organism level and their functional role in the resistance of plants to pests and pathogens (Dolatabadian et al. 2017).

1.11 Research aims

The primary objective of this thesis is to contribute to the pool of knowledge on stability and fertility in allohexaploid *Brassica,* and to create a fertile and stable new species for agriculture. Traditional cytogenetics, phenotypic trait measurements and pollen viability/seed set have previously been used to investigate meiosis and fertility in allohexaploid hybrids. However, little is known about genotypic differences in these traits, or the genetic and genomic factors responsible for stability in *Brassica* allohexaploid hybrids. By utilising a combination of cytological techniques and advances in *Brassica* genomics such as the availability of SNP arrays for high throughput genotyping, I hope to obtain new information about homoeologous recombination and genetic control in novel *Brassica* allohexaploid hybrids.

In chapter 3, I aimed to investigate segregation for fertility and meiotic stability in novel *Brassica* species in a homozygous allohexaploid "A2 population" and in a heterozygous allohexaploid "H2 population". For this purpose, cytogenetic, pollen fertility and phenotypic and statistical analysis were applied. Fertility and meiotic stability assessment were also determined within genotypes and progeny sets of the H2 population.

In chapter 4, I aimed to determine the genetic identity, fertility and meiotic behaviour in two generations of allohexaploid "microspore-derived lines" (MDL1 and MDL2). Combining classical cytogenetics and the *Brassica* 60K Infinium Illumina array to produce high

throughput SNP genotyping data, I aimed to test hypotheses relating to genetic integrity of microspore derived lines in meiotically unstable interspecific hybrids.

In chapter 5, I aimed to create new trigenomic hybrids through interspecific hybridisation between allotetraploid *Brassica juncea* and wild C genome species. I used embryo rescue to overcome hybridisation barriers and used flow cytometry, pollen fertility and phenotypic studies to confirm hybridity of the hybrids. Further, colchicine treatment of the novel ABC hybrids was done with an aim to double the chromosomes, thus creating a new, genetically diverse allohexaploid (AABBCC) *Brassica* species.

Chapter 2: Materials and methods

2.1 Carnoy`s II solution

Carnoy's II solution was prepared according to the protocol described in Singh (2002).
The solution is 10% glacial acetic acid, 30% chloroform and 60% ethanol (95 to 100%).
An important note: fresh Carnoy`s II solution was prepared each morning before sampling by mixing the ingredients in a 1 L Schott bottle inside a fume hood.

2.2 Acetocarmine

The stain acetocarmine (1%) was prepared according to the protocol described in Singh (2002). The preparation of the stain involved boiling 100 mL 45% acetic acid in 55 ml distilled water under a fume hood, followed by adding 1 g carmine powder to the boiling 45% acetic acid. This was then boiled for 5 to 10 minutes, with occasional stirring, until the colour becomes dark red. The solution was filtered into a coloured bottle, and stored at 4°C.

2.3 Sterile distilled water

Sterile distilled water was prepared by autoclaving in 1 L Schott bottles.

2.4 Ethanol (70%)

Up to 100 ml of 70% ethanol was prepared by mixing 70 ml of absolute ethanol and 30 ml of sterile distilled water or milliQ water.

2.5 Sodium Hypochlorite (10%)

Up to 100 ml of 10% sodium hypochlorite was prepared by diluting 10 ml of Sodium hypochlorite (commercial JIK) in 90 ml sterile distilled water or milliQ water.

2.6 Propidium Iodine

Up to 150 µL of propidium iodide (PI) stain solution was prepared as described in Cousin et al. (2009). A total of 5.68 g Na_2HPO_4, 10 mL 10X stock (100 mM sodium citrate, 250 mM

sodium sulphate) and 3.8 mL PI (1.34 mg/mL) were mixed and made up to 100 ml using sterile distilled water or milliQ water and stored in a coloured bottle at 4^{o}C.

2.7 DNA extraction

For the purposes of obtaining good quality DNA, free from shearing and contaminants, the DNeasy® Plant mini kit (QIAGEN©) was used for DNA isolation according to the manufacturer's instructions.

2.8 Agarose gel preparation

Agarose gels were prepared using 1X TAE buffer, 1% Agarose and ethidium bromide at 0.5 µg/mL. The mixture was heated and brought to boil in a microwave oven, stirred to mix well and cooled under a running tap. The mixture was then gently poured into a gel casting tray set up with combs and left to settle.

The 1X TAE buffer was prepared by mixing 242 g Tris Base and 18.96 g EDTA-$Na_{2,}$ the mixture was dissolved in 800 ml milliQ water and pH adjusted to 8.0 with glacial acetic acid. The solution was then made up to 1 L and autoclaved.

2.9 Gel electrophoresis

Gel electrophoresis was carried out to separate DNA fragments based on the size of the fragments. Prior to electrophoresis, 6X loading dye, containing the dyes bromophenol blue and xylene cyanol FF (Thermo Scientific ©), was added to the sample at a concentration of 1X. The Agarose gel was then cast in gel trays prior to running in a gel tank filled with 1X TAE buffer. The voltage varied from 80 to 100 V and a charge of up to 400 milliampere was used. The gel was run from 60 – 90 minutes or until the DNA had migrated sufficiently along the gel.

After the DNA has been electrophoresed on the agarose gel, the gel is imaged using a gel documentation system "Bio Capt" (Vilber Lourmat Copyright). The gel doc system uses UV

light which causes the ethidium bromide bound to the DNA to fluoresce. The images are then visualised on the computer.

2.10 DNA quality and quantity assurance

Extracted DNA samples were run on 1% TAE-Agarose gels to determine the quality. The quality of each DNA can be determined by observation of Agarose gel image, for the presence of, or lack of, smears/shearing. Additionally, the intensity from each DNA sample is compared to a known size ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder) to determine the concentration.

2.10.1 QUBIT values

DNA quantity was also verified using a QUBIT 3.0 Fluorometer (Life Technologies) according to the manufacturer's instructions.

2.11 DNA normalisation

DNA was normalised for Infinium Illumina 60K SNP array genotyping. This was done by diluting the DNA using sterile distilled water to the desired concentration of approximately 5 µg. The DNA was run on a 1% agarose gel for confirmation.

2.12 Data analysis

Data analysis was performed using R statistical programming language (R Core Team). ANOVA and Tukeys` highly significant differences test (HSD) were used while the function "hclust" with a Euclidean distance matrix in R library "pvclust" were used for hierarchical cluster analysis.

2.13 SNP genotyping analysis

High throughput SNP genotyping was done using the Illumina Infinium *Brassica* 60K SNP array (Illumina Inc., San Diego, USA) according to the manufacturer's instructions.

Genotyping data analysis was done using Illumina`s Genome Studio software http://support.illumina.com/array/array software/genome studio/downloads.html.

Application of cluster files and determination of copy number variation was performed as outlined in Mason et al. (2015).

2.14 Flow cytometry analysis

Young leaf tissue (5 mg) samples were collected in 1.2 ml strip tubes containing a 3 mm tungsten carbide bead and then placed on ice. The nucleic suspension prepared for flow cytometry analysis following the protocols and methods described in Cousin et al. (2009). Flow cytometry was carried out at the Centre for Microscopy and Characterisation Analysis (CMCA) at the University of Western Australia, Perth, Australia.

2.15 Ovule rescue media

Ovule rescue media was prepared for wide hybridisation. The media consisted of MS medium (Murashige and Skoog 1962), 100 $mg.l^{-1}$ of glutamine, 25 $mg.l^{-1}$ asparagine, 10 $mg.l^{-1}$ cysteine, 100 $mg.l^{-1}$ inositol, 250 $mg.l^{-1}$ casein hydrolysate, 30 $g.l^{-1}$ sucrose, 0.25 $mg.l^{-1}$ 6-benzyl aminopurine (BAP), 0.25 $mg.l^{-1}$ kinetin, 1 $mg.l^{-1}$ indole acetic acid (IAA), 100 $mg.l^{-1}$ sodium nitroprusside and 2 $g.l^{-1}$ gelrite. Ovule rescue media was autoclaved and allowed to cool and settle for 24 hours before dispensing into petri-dishes for use. Allowing the media to settle before use also allowed detection of any contamination, if present. The plant hormones i.e. 6-benzyl aminopurine (BAP) and indole acetic acid (IAA) were filter sterilized and added to the ovule rescue media after autoclaving.

2.16 Regeneration media

Germinating plants were subsequently cultured in regeneration media. The media consisted of 4.4 g of MS medium (Murashige and Skoog 1962), 100 $mg.l^{-1}$ of glutamine, 25 $mg.l^{-1}$ asparagine, 10 $mg.l^{-1}$ cysteine, 100 $mg.l^{-1}$ inositol, 250 $mg.l^{-1}$ casein hydrolysate, 30 $g.l^{-1}$ sucrose, 0.25 $mg.l^{-1}$ 6-benzyl aminopurine (BAP), 1 $mg.l^{-1}$ indole acetic acid (IAA), 100 mg/l sodium nitroprusside and 2 $g.l^{-1}$ gelrite, 0.5 $mg.l^{-1}$ gibberellic acid (GA_3) and 0.5 $mg.l^{-1}$ kinetin.

Regeneration media was autoclaved and allowed to cool and settle for 24 hours before dispensing into petri-dishes for use. Allowing the media to settle before use also allowed detection of any contamination, if present. The hormones i.e. gibberellic acid (GA_3), 6-benzyl aminopurine (BAP) and indole acetic acid (IAA) were filter sterilized and added to the regeneration media after autoclaving.

2.17 Multiplication media

The plants were cultured in a series of multiplication media composed of 4.4 g MS medium (Murashige and Skoog 1962), 30 $g.l^{-1}$ sucrose supplemented with 0.2 $mg.l^{-1}$ 6-benzyl aminopurine (BAP), 1 $mg.l^{-1}$ indole acetic acid (IAA), 0.5 $mg.l^{-1}$ gibberellic acid (GA_3) and 2 $g.l^{-1}$ Gelrite. The media was autoclaved and allowed to cool and settle for 24 hours before dispensing into petri-dishes for use. Allowing the media to settle before use also allowed detection of any contamination, if present. The plant growth hormones i.e. gibberellic acid (GA_3), 6-benzyl aminopurine (BAP) and indole acetic acid (IAA) were filter sterilized and added to the multiplication media after autoclaving.

The ovule rescue media, regeneration media and multiplication media protocols were obtained from Dr. Chaya Atri (Punjab Agricultural University, India.).

2.18 Colchicine media

Colchicine chemical treatment is used in tissue culture to arrest formation of spindle fibres during meiosis, thus inducing polyploidy. In order to develop allohexaploid buds, interspecific hybrids were cultured on colchicine-containing media (Yao et al. 2012) which consisted of MS medium (Murashige and Skoog 1962) supplemented with 0.1 $g.l^{-1}$ colchicine, 0.25 $mg.l^{-1}$ naphthalenacetic acid (NAA) and 1.5 $mg.l^{-1}$ 6-benzyl aminopurine (6-BAP) for 10 – 14 days. The buds were then transferred from the colchicine media to fresh medium without any colchicine until new plants regenerated. The plant growth hormones were filter sterilized and added to the media after autoclaving.

Different serial dilutions treatments were made i.e. 0.05%, 0.1%, 0.15%, 0.2%, 0.25 (w/v) by diluting 1% colchicine stock solution (1 g in 100 ml) in sterile distilled water or milliQ water

(Pradhan et al. 2010). The treatments were used to induce chromosome multiplication in trigenomic ABC hybrids to create AABBCC allohexaploid hybrids.

2.19 Root media

Rooting media (Yao et al. 2010) was prepared by using MS medium (Murashige and Skoog 1962) supplemented with 1.5 $mg.l^{-1}$ 6-benzyl aminopurine (6-BAP), 0.25 $mg.l^{-1}$ and naphthalenacetic acid (NAA). The plant growth hormones were filter sterilised and added to the media after autoclaving.

Chapter 3: Segregation for fertility and meiotic stability in novel *Brassica* allohexaploids

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3.1 Introduction

The genus *Brassica* is one of 51 genera in the tribe Brassiceae belonging to the crucifer family and is the most economically important genus within this tribe (Rakow 2004). Vegetable *Brassica* species include *B. oleracea* and *B. rapa*, while the seeds of *B. nigra* and *B. juncea* are also used as a condiment and *B. rapa*, *B. juncea*, *B. carinata* and *B. napus* are used as oilseeds. *Brassica* species are rich in dietary fibre, vitamin C, and phytosterols, and contain beneficial anti-carcinogenic compounds. In addition, the use of *Brassica* species as renewable raw materials has attracted growing interest for the biofuel and chemical industries (Chen et al. 2011).

The *Brassica* genus is an interesting model for allopolyploid formation in agricultural crops, as six agriculturally significant species share a genomic and evolutionary relationship. The predecessors of the diploid species *B. rapa* (A genome, 2n = 20, Chinese cabbage and turnip), *B. nigra* (B genome, 2n = 16, black mustard) and *B. oleracea* (C genome, 2n = 18, cabbage, cauliflower, broccoli) are hypothesised to have given rise to the allotetraploid species *B. juncea* (A and B genome, 2n = 36, leaf mustard, Indian mustard), *B. napus* (A and C genome, 2n = 36, oilseed rape, canola) and *B. carinata* (B and C genome, (2n = 34, Ethiopian mustard) through pairwise hybridisation. This relationship is referred to as the *Brassica* U's triangle and is an evolutionary example of allopolyploidy (Morinaga 1934; U 1935).

Polyploidisation has played a major role in plant evolution and speciation, allowing adaptation over a wide ecological landscape and increased "hybrid vigour" relative to progenitor diploids (Ramsey and Schemske 1998; Leitch and Leitch 2008; Song et al. 2012). For example, tetraploid cotton is a successful polypoid and is preferred in fibre production for its longer, stronger and finer fabric compared to its diploid relatives (Udall and Wendel 2006). Similarly,

successfully developing an allohexaploid *Brassica* (2n = AABBCC) could benefit from the positive effects of polyploidisation over its allotetraploid relatives for agricultural benefit (Chen et al. 2011).

In the past, attempts to create *Brassica* allohexaploids involved induction of somatic chromosome doubling of triploid ABC interspecific hybrids to form AABBCC allohexaploids, with *B. rapa* (2n = AA) × *B. carinata* (2n = BBCC) being the most common cross combination (Iwasa 1964; Pradhan et al. 2010). The studies conducted mainly investigated the use of allohexaploid *Brassica* as a bridge to transfer useful traits, such as disease resistance, seed colour and cytoplasmic male sterility, into cultivated allotetraploids, and to resynthesize the allotetraploid species from their diploid progenitors; however, these lines were often characterised by chromosomal instability and poor seed set (Sjödin and Glimelius 1989; Arumugam et al. 1996; Meng et al. 1998; Li et al. 2004). Howard (1942) found that *Brassica* crosses from *B. rapa* × *B. carinata* showed improved fertility over a few generations, whilst Iwasa (1964) found low fertility in hybrids up to the fifth generation using similar crosses. Recent studies with *Brassica* allohexaploids from different genotype combinations, including the *B. rapa* and *B. carinata* crosses, suggest increased fertility and stability may arise in subsequent generations (Tian et al. 2010; Zhou et al. 2016).

An alternate approach to create allohexaploid *Brassica* involves production of unreduced gametes, rather than somatic doubling, to increase the ploidy level (Mason et al. 2010). The allotetraploid U's Triangle species (U 1935) can be crossed in a pairwise fashion to produce trigenomic hybrids with the unbalanced genome complements AABC, BBAC and CCAB. Hybrids were found to produce various frequencies of unreduced gametes (gametes with the somatic chromosome number; i.e. chromosome complements AABC, BBAC, and CCAB) which were hypothesised to be passed on to the next generation in a round of crossing with the third allotetraploid species to produce allohexaploid *Brassica* AABBCC). This approach was only partially successful; one near-allohexaploid hybrid was produced and characterised (Mason et al. 2012). Despite the various attempts in creating an allohexaploid *Brassica* species, a completely stable allohexaploid *Brassica* (AABBCC genome 2n = 54) remains elusive, and fertility and meiotic stability over subsequent generations have only been examined in a few studies (e.g. Tian et al. 2010; Zhou et al. 2016).

Chromosome behaviour during meiosis must be strictly controlled to facilitate correct segregation of chromosomes into daughter cells, which can be a challenge in polyploids which contain more than two pairs of chromosomes (Cifuentes et al. 2010b). A proper sorting-out mechanism for these chromosomes is therefore necessary to avoid illegitimate associations that would otherwise lead to aneuploidy. Information on factors influencing meiotic stability in allohexaploid *Brassica* remains sparse, and more studies regarding stability and fertility of hexaploids are necessary to help establish a stable and fertile allohexaploid species. In this study fertility and meiotic stability were assessed in homozygous allohexaploid A2 progeny produced through the cross *B. rapa* × *B. carinata*, and in heterozygous allohexaploid H2 progeny derived from different genotypes in the cross (*B. napus* × *B. carinata*) × *B. juncea* .

3.2 Materials and methods

3.2.1 Plant material generation

Interspecific hybridisation between *B. carinata* (Indian line PGR 16789) × *B. rapa* (*PAK* 85835) was performed by hand pollination in Punjab Agricultural University (PAU), Ludhiana, India. From a total of 308 buds pollinated, 42 pods and 15 putative hybrid seeds were obtained, resulting in seven viable triploid F_1 plants. Chromosome doubling to induce polyploidy was done using 0.2% colchicine in 1% DMSO on the axillary meristem at the four-leaf stage, and three allohexaploid A1 generation plants obtained. The A1 lines were confirmed to have 2n = 27 chromosome complements. These allohexaploid plants were then self-pollinated to produce A2 seeds.

The *Brassica* allohexaploid (H1) population was produced by the cross (*B. napus* × *B. carinata*) × *B. juncea* at The University of Queensland, Brisbane following procedures outlined in Mason et al. (2012). The *B. juncea* parent genotypes were "JN9-04", a self-pollinated single plant selection by Janet Wroth (The University of Western Australia, Perth, Australia) from near canola quality *B. juncea* line "JN9" supplied by Wayne Burton (Department of Primary Industries, Horsham, Victoria, Australia), and inbred line "Purple Leaf Mustard" (donated by Huazhong Agricultural University, Wuhan, China). The *B. napus* parent genotypes were Australian canola lines "Boomer" (doubled-haploid (DH); Canola Breeders Western Australia), Surpass400_024DH (Canola Breeders Western Australia), "Lynx_037DH" (Canola Breeders Western Australia) and "Ag-Spectrum" (sourced from the Australian Grains Genebank (AGG)), while the *B. carinata* genotypes were DH selections from Ethiopian lines "94024" and "1923" (sourced from the AGG).

A total of 146 H2 seeds from 12 H1 plants (described in Mason et al. 2016) were planted under field conditions at PAU India, of which 69 H2 plants germinated and were characterised. The genotype combinations in the H2 population (Table 3.1) are hereafter referred to as "G1", "G2", "G3" and "G4"). Phenotype data for the parent genotypes was not available; however, a ten-plant average for fertility and phenotypic data was collected and available for comparison for the *B. carinata* "PC5", *B. rapa* "TL-17", *B. juncea* "RLC-1" and *B. napus* "GSC-5"

genotypes as control cultivar samples. The control species were all commercial cultivar varieties.

Table 3.1 Summary of the experimental allohexaploid H2 population showing genotype information

H1 parent	**No. planted**	***B. napus* genotype**	***B. carinata* genotype**	***B. juncea* genotype**	**Genotype combination**	**No. H2 progeny**
H1-006	28	"Boomer"	"94024.2_02DH"	"JN9-04"	G1	10
H1-014	4	"Boomer"	"94024.2_02DH"	"JN9-04"	G1	1
H1-015	14	"Boomer"	"94024.2_02DH"	"JN9-04"	G1	4
H1-016	3	"Boomer"	"94024.2_02DH"	"JN9-04"	G1	2
H1-020	10	"Boomer"	"94024.2_02DH"	"Purple Leaf Mustard"	G2	5
H1-022	32	"Boomer"	"94024.2_02DH"	"Purple Leaf Mustard"	G2	17
H1-023	26	"Boomer"	"94024.2_02DH"	"Purple Leaf Mustard"	G2	17
H1-040	2	"Boomer"	"94024.2_02DH"	"Purple Leaf Mustard"	G2	2
H1-044	2	"Boomer"	"94024.2_02DH"	"Purple Leaf Mustard"	G2	2
H1-052	4	"Ag-Spectrum"	"94024.2_02DH"	"Purple Leaf Mustard"	G3	2
H1-058	8	"Ag-Spectrum"	"94024.2_02DH"	"Purple Leaf Mustard"	G3	6
H1-087	1	"Lynx_37DH"	"1923.3.2_01DH"	"JN9-04"	G4	1

3.2.2 Phenotypic characterisation

Phenotypic data for the H2 allohexaploid population (69 plants) and the A2 allohexaploid population (100 plants) were collected in the *Brassica* fields at PAU between April and May 2015. Data collected included plant height (cm), number of pods on main shoot, number of seeds on main shoot, total seed set and the seeds per 10 pods.

3.2.3 Pollen fertility and seed set

Ripe floral buds (near opening) were collected from *Brassica* genotypes grown at PAU, Ludhiana, India. Pollen studies were conducted with squashed anthers in 1% acetocarmine (prepared as described in section 2.2) on glass microscope slides and observed under a compound light microscope. Plump, darkly stained pollen was assumed to be viable, while unstained and/or shrivelled pollen were considered as unviable. A total of 300 pollen grains were scored for each sample in both allohexaploid progenies. Fertility and phenotypic data were recorded for reference control cultivars, A2 and H2 allohexaploid progenies. Self-pollination was encouraged by enclosing racemes in bags.

3.2.4 Meiotic chromosome observations

Floral buds were fixed in Carnoy's II solution (ethanol: chloroform: acetic acid 6:3:1) (prepared as described in section 2.1) for 72 hours and stored in 70% ethanol (prepared as described in section 2.4) at 4^{o} C. Anthers were squashed and stained in a drop of 1% acetocarmine solution on glass microscope slides. Sixty-nine plants from the H2 allohexaploid population and 100 plants from the A2 allohexaploid population were assessed for chromosome number. Five to twenty pollen mother cells (mode ten) were observed for each plant. Observations of the pollen mother cells (PMCs) were performed at metaphase I, and anaphase I stages, and images captured using Cytovision 4.2 software Leica Biosystems. Chromosome meiotic images were observed using an Olympus BX 61 compound bright field microscope using Cytovision software. Univalent, bivalent and multivalent associations were assessed.

3.2.5 Data analysis

ANOVA analysis, Tukey's Honest Significant Differences test, Pearson's product moment correlations and boxplots were carried out using R version 3.2.2 (The R Foundation for Statistical Computing 2015). The (aov), (summary) R commands were run to determine Analysis of Variance (ANOVA) for the means of meiotic characteristics, chromosome numbers, fertility and plant traits per genotype and progeny sets. Tukey's Honest Significant Differences test (Tukey HSD) was used to establish significant differences between genotypes and progeny sets for each trait.

3.3 Results

3.3.1 Chromosome numbers and meiotic behaviour in the A2 and H2 populations

In the A2 population the number of univalents (unpaired chromosomes) at metaphase I averaged 3 per pollen mother cell (PMC), ranging from 1 – 7 chromosomes, while the average number of bivalents (chromosome pairs) was 19 with a range of 12 – 27 chromosomes (Figure 3.1A, 3.1B and 3.1C). The mean meiotic configuration was 2I + 19II with an average chromosome number estimate of 41 chromosomes (Figure 3.2A). In the H2 population the average number of univalents (unpaired chromosomes) was 2 per PMC with a range of 0 – 2, while the average number of bivalents was 24 chromosomes (Figure 3.1D, 3.1E and 3.1F). The mean meiotic configuration was 2I + 24II with an average estimate of 49 chromosomes (Figure 3.2B).

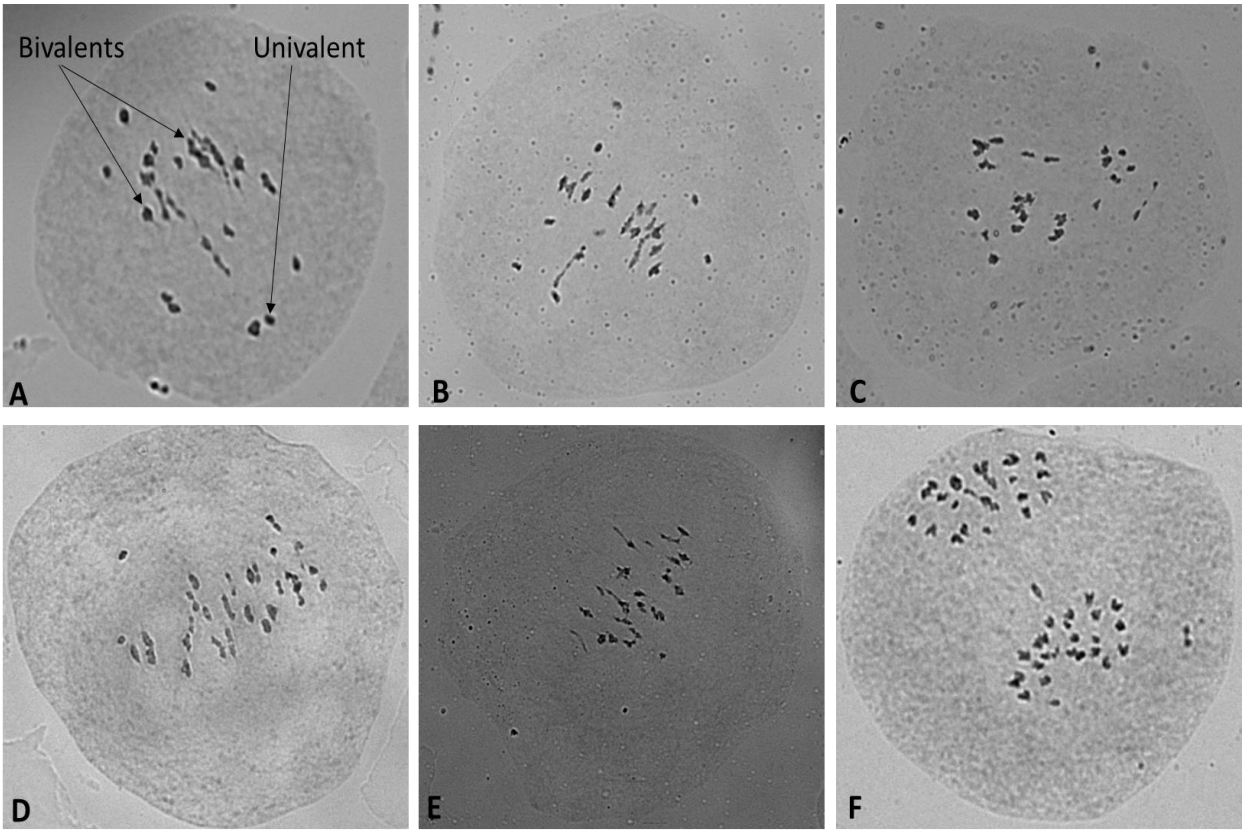


Figure 3.1 Meiotic configurations in the A2 population (*B. rapa* × *B. carinata* allohexaploids) A. 23II, 1I at metaphase-I; B. 18II, 7I at metaphase-I; C. 22I, 22I at anaphase; and in the H2 population (*B. napus* × *B. carinata)* × *B. juncea* allohexaploids) D. 24II, 2I at metaphase-I; E. 26II, 1I at metaphase-I F. 24I, 26I at anaphase-1 (Magnification using 100 × objective lens)

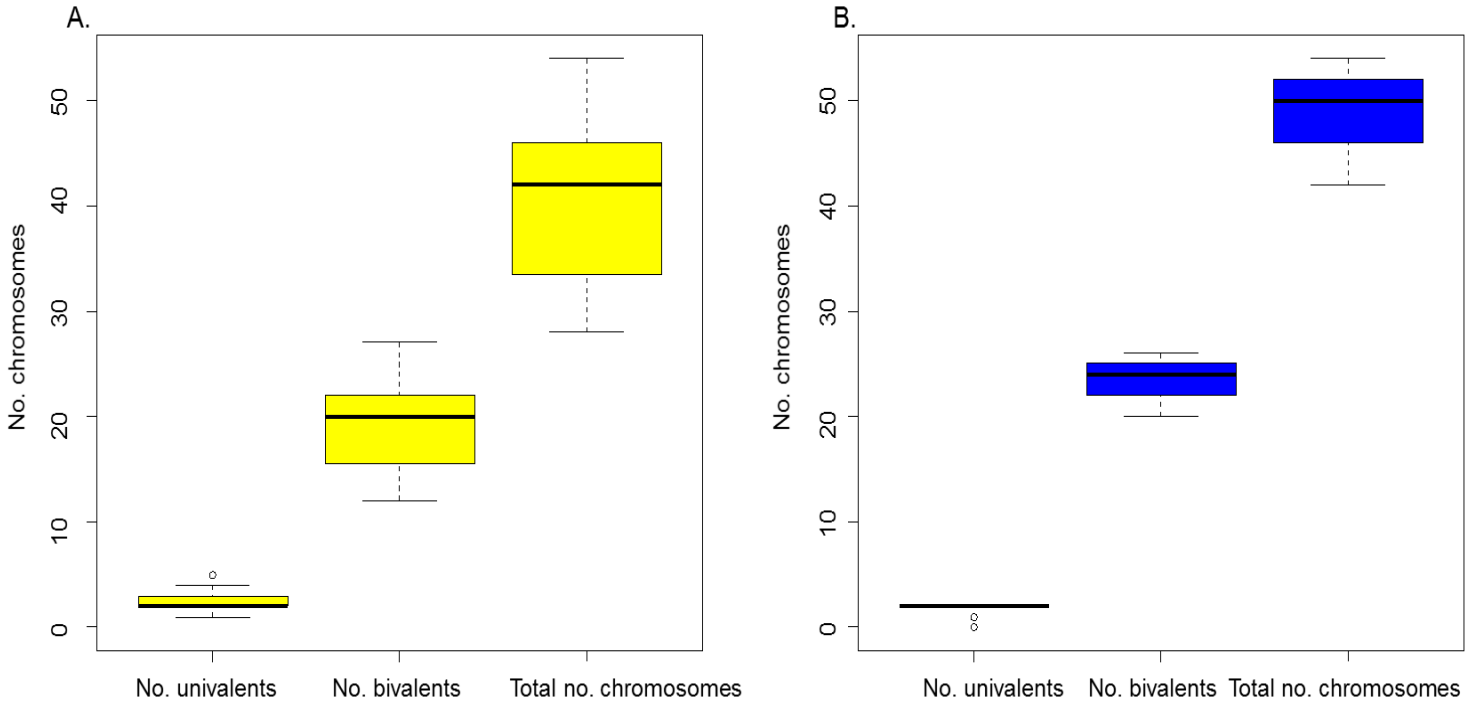


Figure 3.2 Chromosome number and average meiotic behaviour in A. An allohexaploid population (A2) derived from crosses between *B. rapa* and *B. carinata* and B. An allohexaploid population (H2) derived from the cross (*B. napus* × *B. carinata)* × *B. juncea*

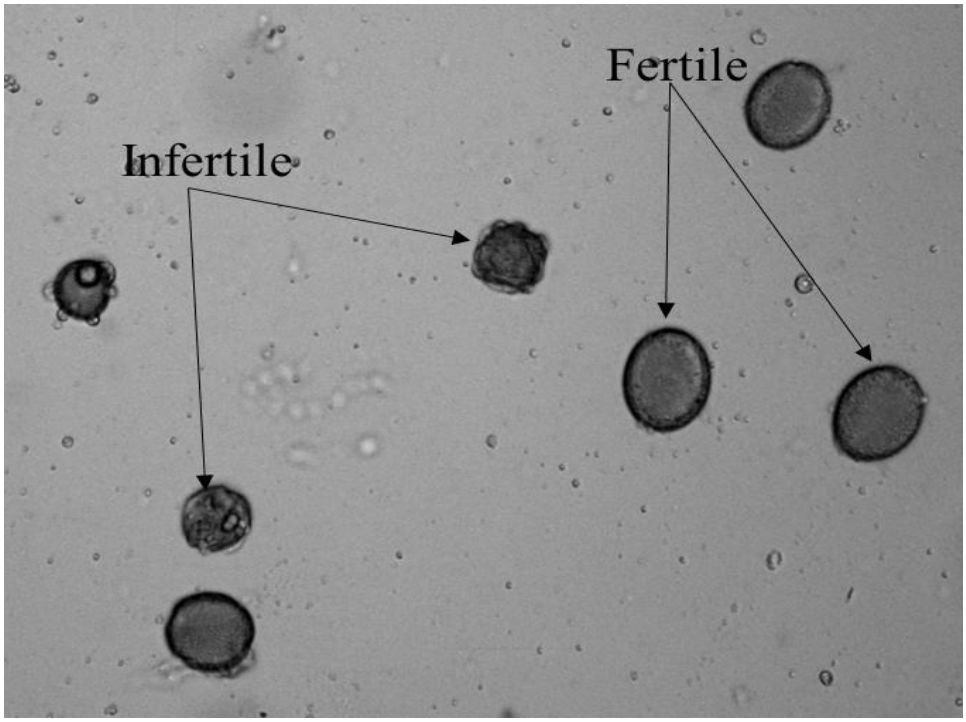


Figure 3.3 Pollen fertility image showing fertile and infertile pollen grains in the H2 allohexaploid population derived from the cross (*B. napus* × *B. carinata*) × *B. juncea* (Magnification using 100 × objective lens)

3.3.2 Fertility estimates and plant height in A2 and H2 population

Pollen fertility (%) (Figure 3.3) in *B. carinata, B. juncea* and *B. napus* control cultivars was all > 90%. Pollen fertility in the A2 population was lower compared to the control cultivars, averaging 13% with a range of 0 - 63% (Figure 3.4A), while average pollen fertility in the H2 population was 64% with a range of 1 - 94 % (Figure 3.4B). Three lines in the H2 population were in the same range as the controls for pollen fertility.

The *B. carinata* and *B. rapa* control cultivars had an average of 38 and 45 pods on the main shoot respectively, which was lower than the average of 52 pods with a range of 14 – 89 pods observed in the A2 population (Figure 3.5A). The number of seeds on the main shoot in *B. carinata* and *B. rapa* controls was 342 and 497 seeds respectively, which was higher than the average of 199 seeds with a range of 12 - 482 seeds in the A2 population. The most fertile plants exceeded the number of seeds produced by the *B. carinata* control cultivar and showed

similar seed numbers to the *B. rapa* control cultivar (Figure 3.5B). The number of seeds per 10 pods was 90 and 110 seeds in *B. carinata* and *B. rapa* controls respectively which was higher than the average of 37, with a range of 5 - 65 seeds, observed in the A2 population (Figure 3.5C). Finally, the plant height of the *B. carinata* and *B. rapa* control was 130 cm and 150 cm respectively, both control cultivars being shorter than the average of 240 cm with a range of 185 – 291 cm in the A2 population (Figure 3.5D).

The *B. carinata*, *B. napus* and *B. juncea* control cultivars for the H2 population had an average of 38, 54 and 43 pods on the main shoot respectively, while the H2 population had an average of 41 pods and a range of 19 - 62 pods. The average number of pods was within the range of the control cultivars, while ten of the plants in the population possessed a higher number of pods than the highest *B. napus* parent (Figure 3.6A). The controls *B. carinata*, *B. napus* and *B. juncea* had 342, 702 and 475 seeds on the main shoot respectively, which were all higher than the average of 105 seeds in the H2 population (range of 10 - 450 seeds) (Figure 3.6B). The average number of seeds per 10 pods in the respective *B. carinata*, *B. napus* and *B. juncea* controls was 90, 130 and 95 seeds respectively, compared to an average of 24 seeds per 10 pods and a range of 2 - 73 seeds in the H2 population (Figure 3.6C). Finally, the average plant height of the *B. carinata*, *B. napus* and *B. juncea* controls was 130, 198 and 138 cm respectively, compared to an average height in the H2 population of 190 cm, with a range of 100 - 248 cm (Figure 3.6D).

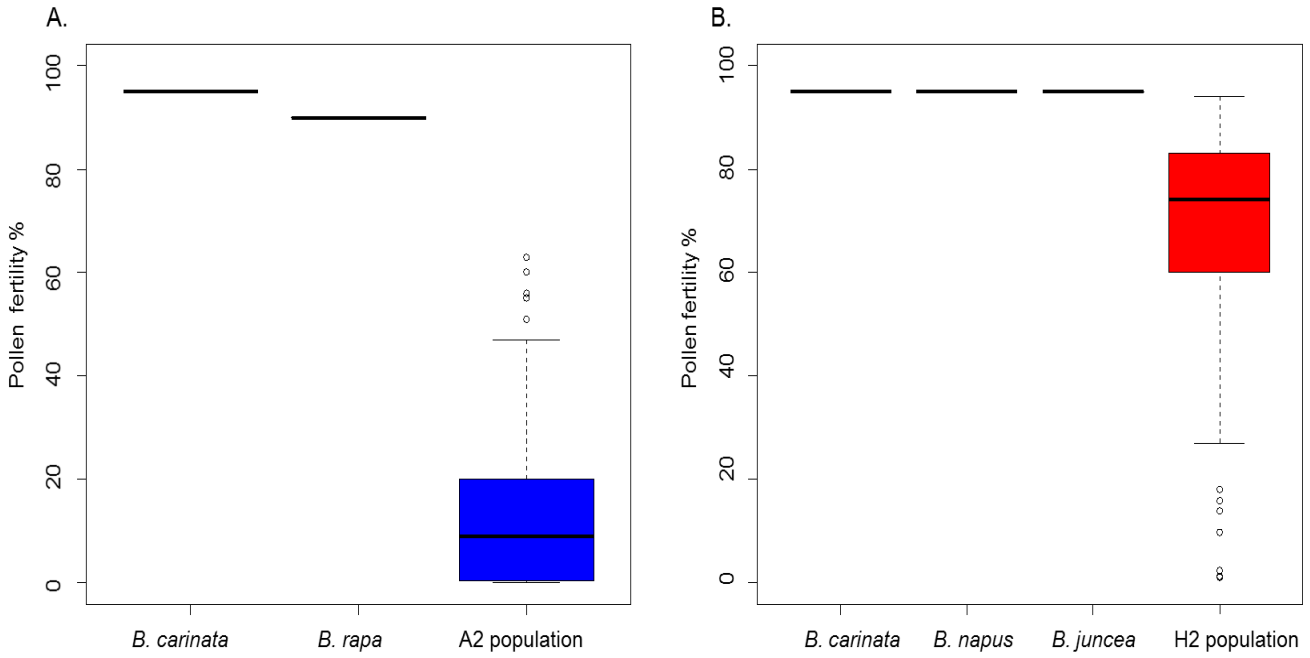


Figure 3.4 Fertility traits in A. An allohexaploid population (A2) derived from crosses between *B. rapa* and *B. carinata* and in B. An allohexaploid population (H2) derived from different genotypes of the cross (B. *napus* × *B. carinata)* × *B. juncea* compared against control cultivar samples *B. carinata* (PC5) and *B. rapa* (TL-17) genotypes in the A2 population and *B. carinata* (PC5), *B. juncea* (RLC-1) and *B. napus* (GSC-5) genotypes in the H2 population

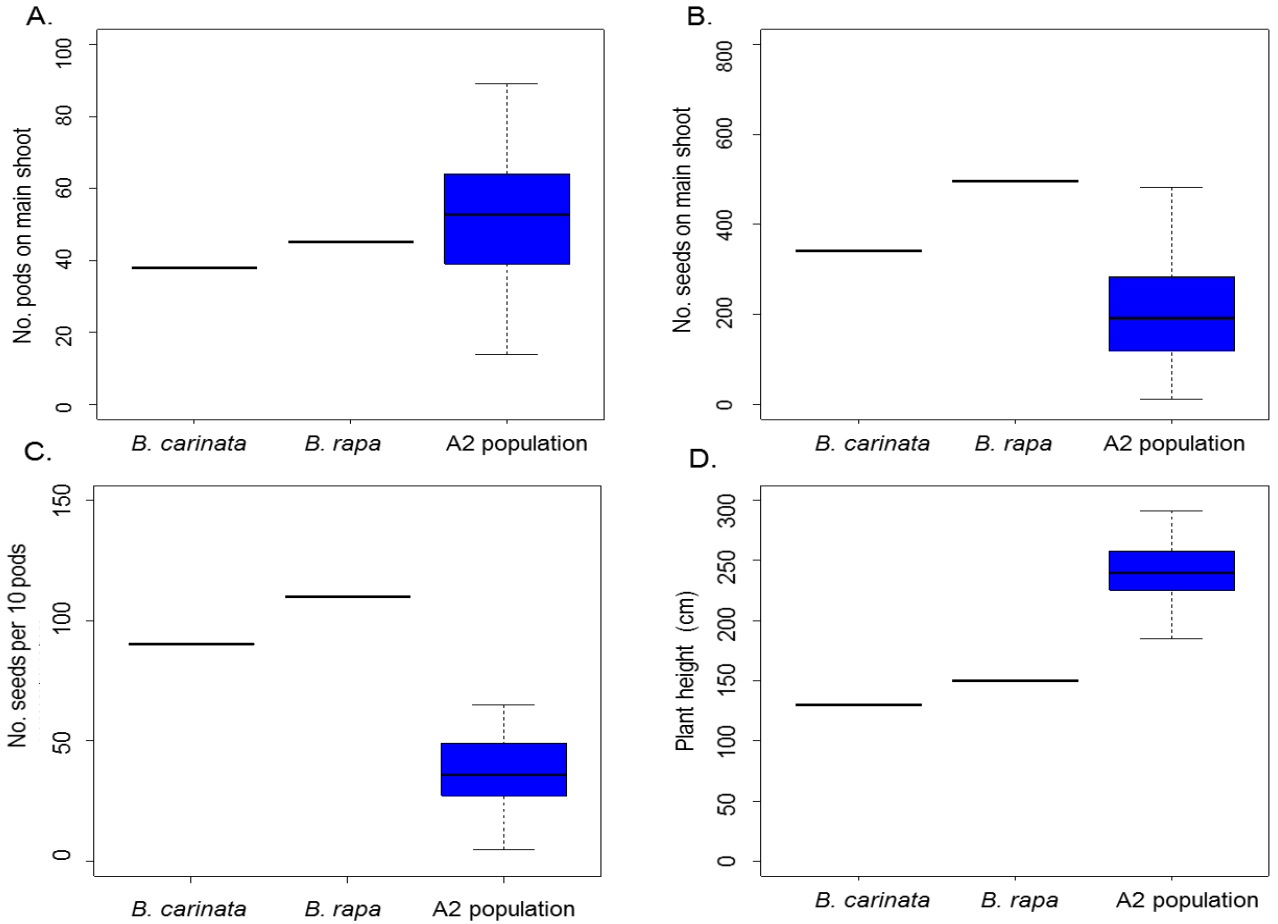


Figure 3.5 Phenotypic traits (A, B, C and D) in an allohexaploid population (A2) derived from crosses between *B. rapa* and *B. carinata* compared against control cultivar samples *B. carinata* (PC5) and *B. rapa* (TL-17)

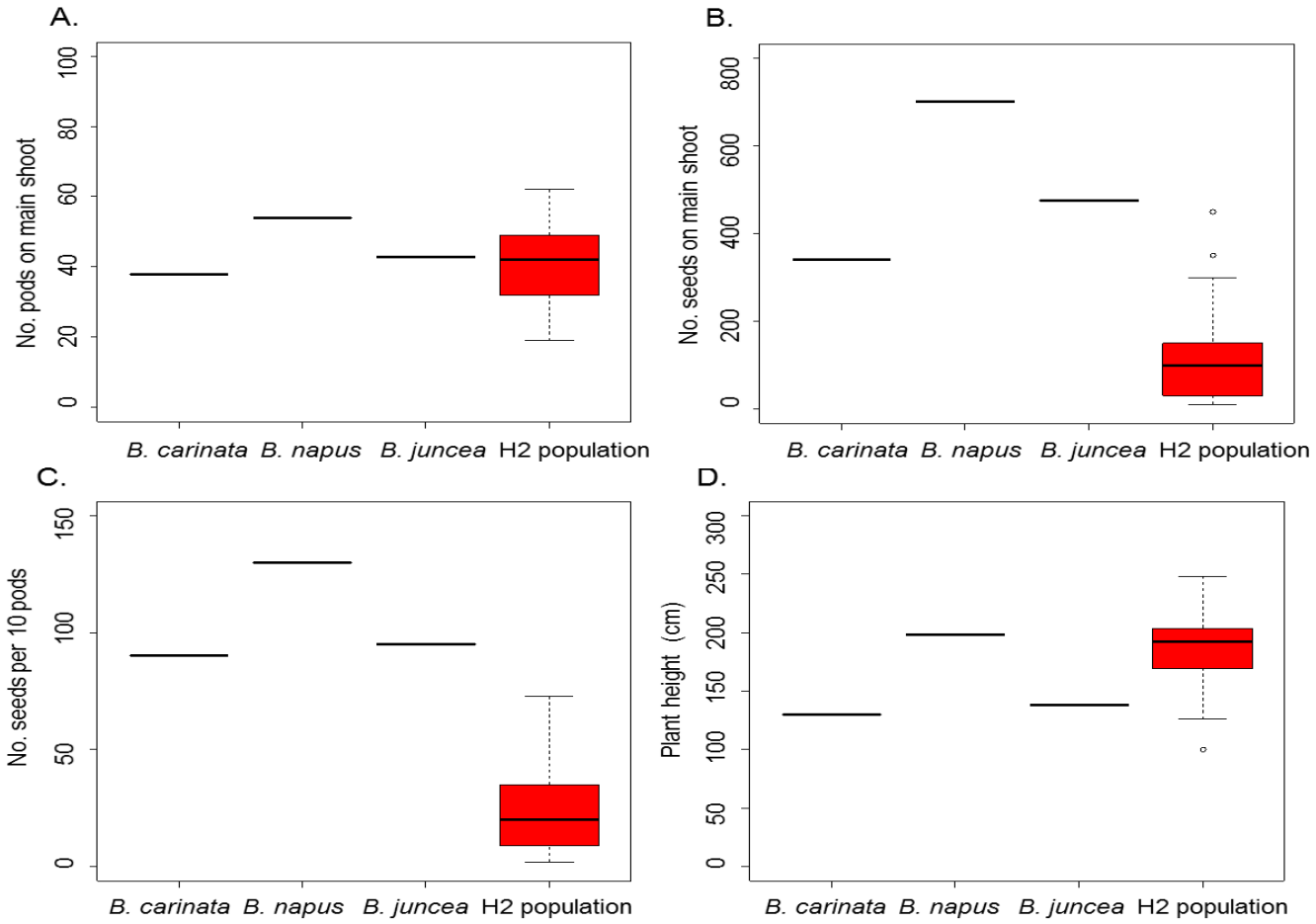
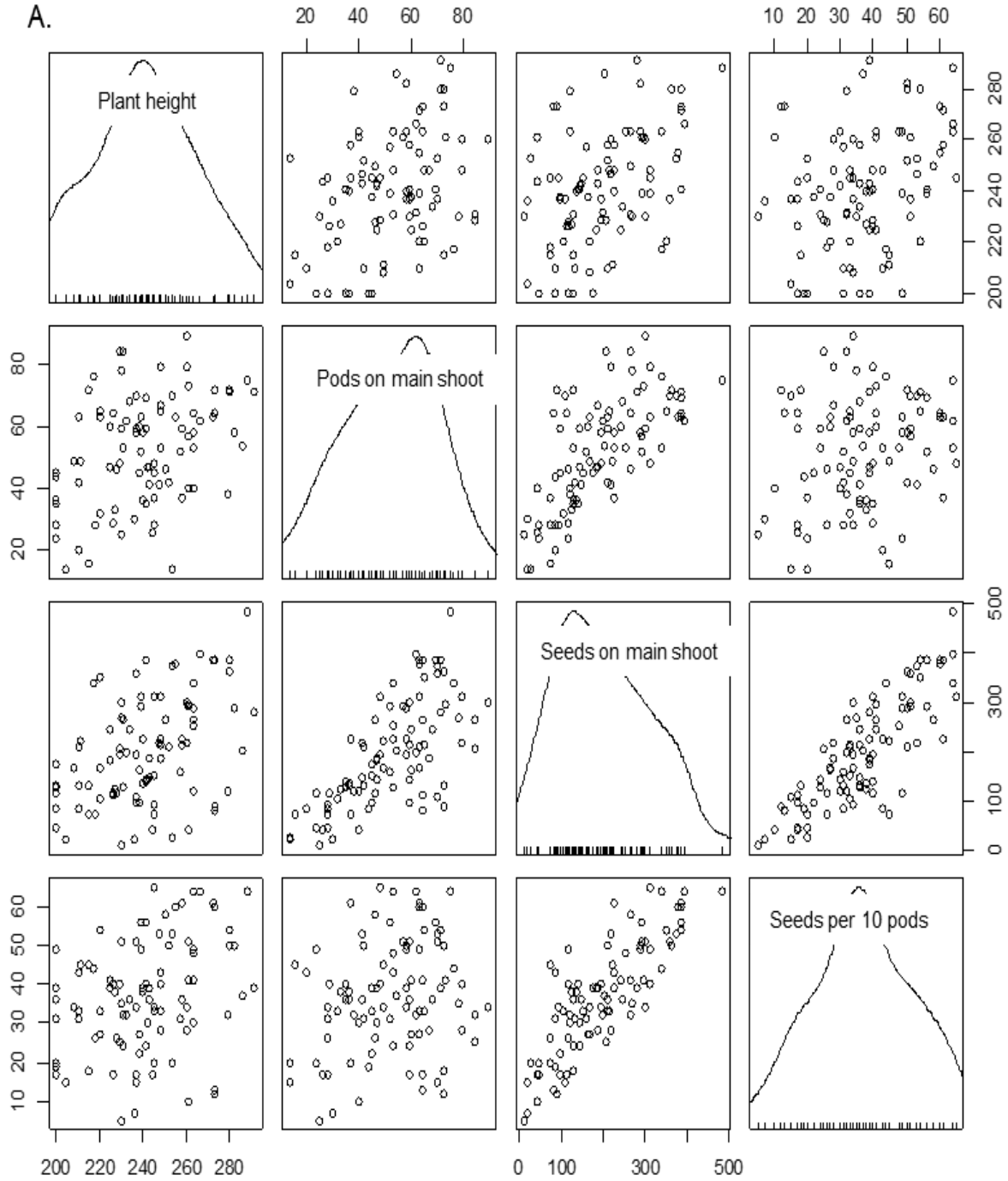


Figure 3.6 Phenotypic traits (A, B, C and D) in an allohexaploid population (H2) derived from the cross *(B. napus* × *B. carinata*) × *B. juncea* compared against control cultivars *B. carinata* (PC5), *B. juncea* (RLC-1) and *B. napus* (GSC-5)

3.3.3 Correlations between chromosome numbers, meiotic behaviour and fertility traits in the A2 and H2 populations

A2 allohexaploid progeny showed a significant positive Pearson's product-moment correlation of r = 0.82 between the number of seeds on the main shoot and seeds per 10 pods (linear regression *p* - value < 0.0001, adjusted R^2 = 0.68). No other traits were significantly correlated in this population (Figure 3.7A). Correlation between various fertility traits and plant height in the H2 allohexaploid progeny showed that the number of seeds on the main shoot and number of seeds per 10 pods were significantly positively correlated with a Pearson's product-moment correlation of r = 0.94 (linear regression *p* - value < 0.0001, adjusted R^2 = 0.8812). No other traits were significantly correlated (Figure 3.7B).


A.
Plant height
Pods on main shoot
Seeds on main shoot
Seeds per 10 pods
20 40 60 80
10 20 30 40 50 60
200 220 240 260 280
20 40 60 80
0 100 300 500
10 20 30 40 50 60
200 220 240 260 280
0 100 300 500

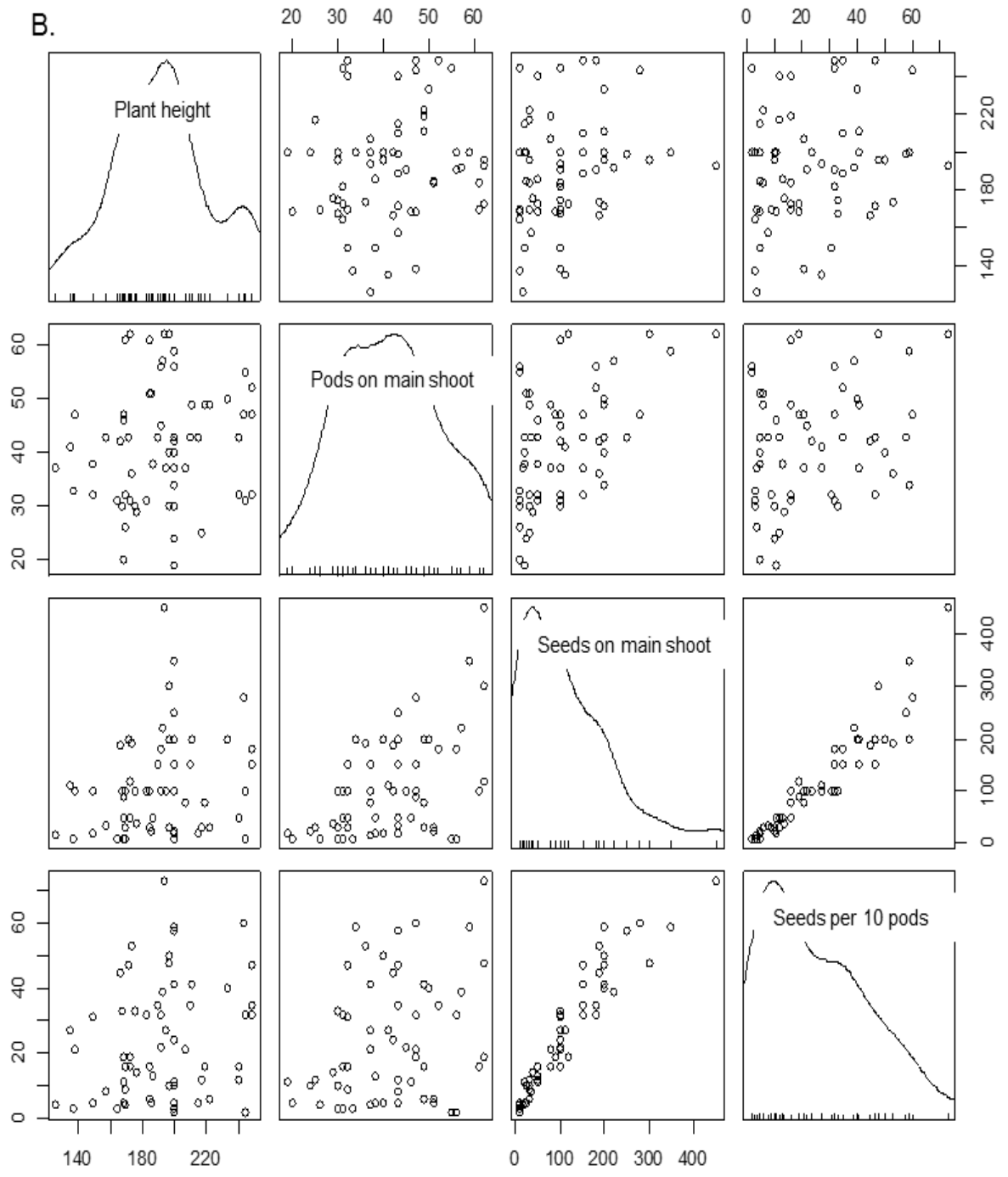


Figure 3.7 Scatter plot matrix showing correlation and distributions of phenotypic traits between A. An allohexaploid population (A2) derived from crosses between *B. rapa* and *B. carinata* and B. An allohexaploid population (H2) derived from the cross (*B. napus* × *B. carinata)* × *B. juncea*

3.3.4 Analysis of variance (ANOVA) for genotypes and progeny set in the H2 allohexaploid population

There were no differences observed between genotypes in the number of univalent per PMC between genotypes in the H2 population. However, significant differences were found between genotypes and the mean number of bivalents *(p* = 0.000017), total number of chromosomes (*p* = 0.00003) and plant height (*p* = 0.000448), (one-way ANOVA; Table 3.2).

Further, post-hoc analysis using Tukey's Honest Significant Differences (HSD) test found significant differences ($p < 0.05$) in means of bivalents between "G1" and "G2" and between "G2" and "G3" genotypes (Figure 3.8A), while significant differences ($p < 0.05$) in the mean number of chromosomes were found between the "G1" and "G2" genotypes and between the "G2" and "G3" genotypes (Figure 3.8B).

Significant differences ($p < 0.05$) in means of plant height using Tukey's HSD test were found only between the "G1" and "G2" genotypes (Figure 3.9). The "G4" genotype had only a single plant and was therefore omitted in this analysis. Bonferroni correction for multiple testing was carried out using $\alpha = 0.05$: $p < 0.0062$.

Table 3.2 ANOVA of chromosomes, meiotic, fertility traits among genotypes and progeny sets in the H2 allohexaploid population derived from the cross (*B. napus* × *B. carinata)* × *B. juncea*. Bonferroni correction for multiple testing at α = 0.05: p < 0.00278

ANOVA (one-way)	**Univalent (*p* - value)**	**Bivalents (*p* - value)**	**Chromosomes (*p* - value)**	**Pollen fertility (*p* - value)**	**Total seed (*p* – value)**	**Seeds per 10 pods (*p* - value)**	**Seeds on main shoot (*p* - value)**	**Pods on main shoot (*p* - value)**	**Plant height (*p* - value)**
Genotypes	0.28	1.7e-05 ***	3.02e-05 ***	0.74	0.374	0.77	0.883	0.644	0.000448 ***
Progeny sets	0.918	0.000375 ***	0.000678 ***	3.02e-05 ***	0.011 *	0.0906	0.238	0.439	4.12e-06 ***

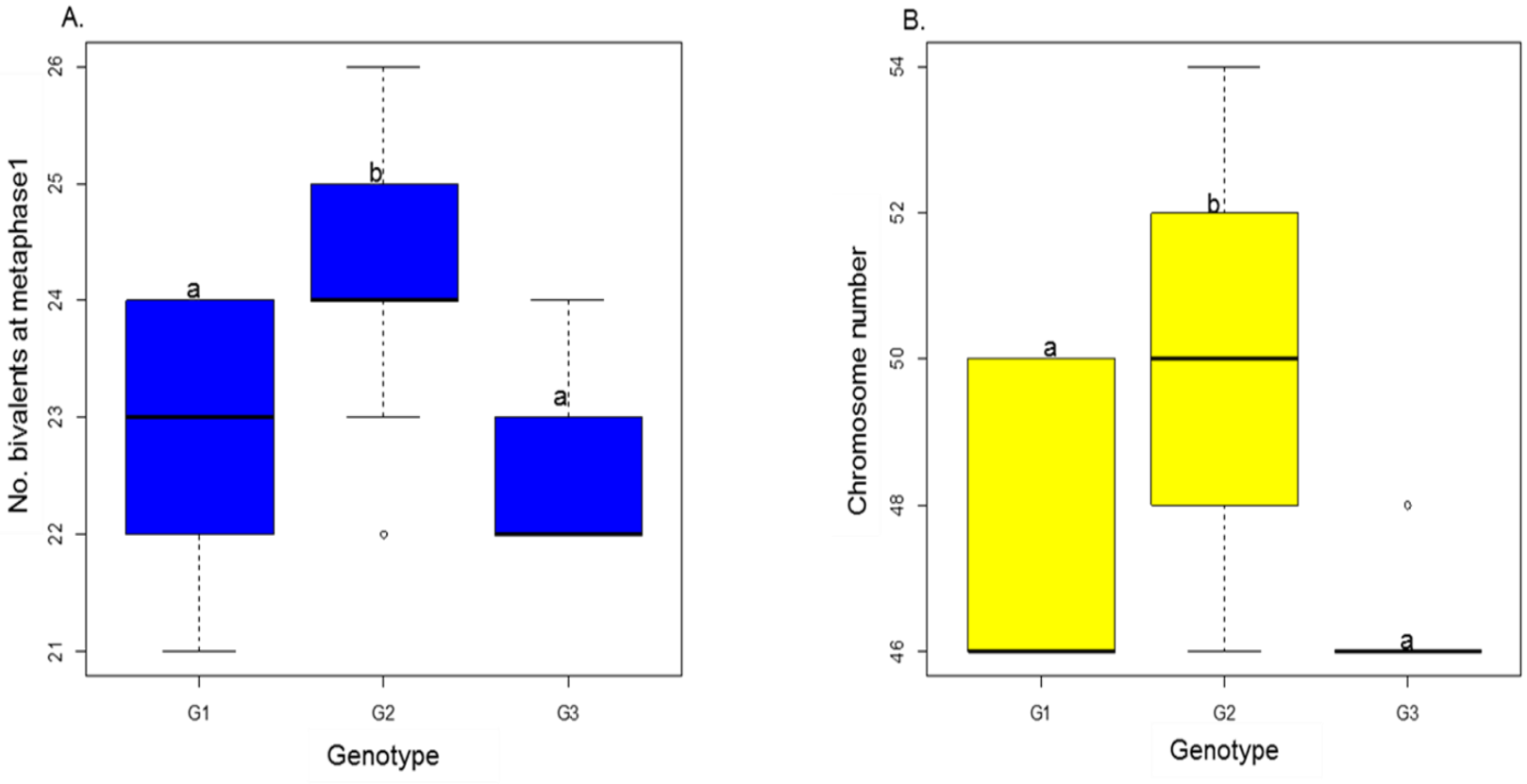


Figure 3.8 The number of A. Bivalents at metaphase I of meiosis B. Chromosome number in the H2 allohexaploid population derived from the cross (*B. napus* × *B. carinata)* × *B. juncea*, showing significant differences between genotypes ($p < 0.05$, one-way ANOVA), ($p < 0.05$, a and b Tukey's HSD)

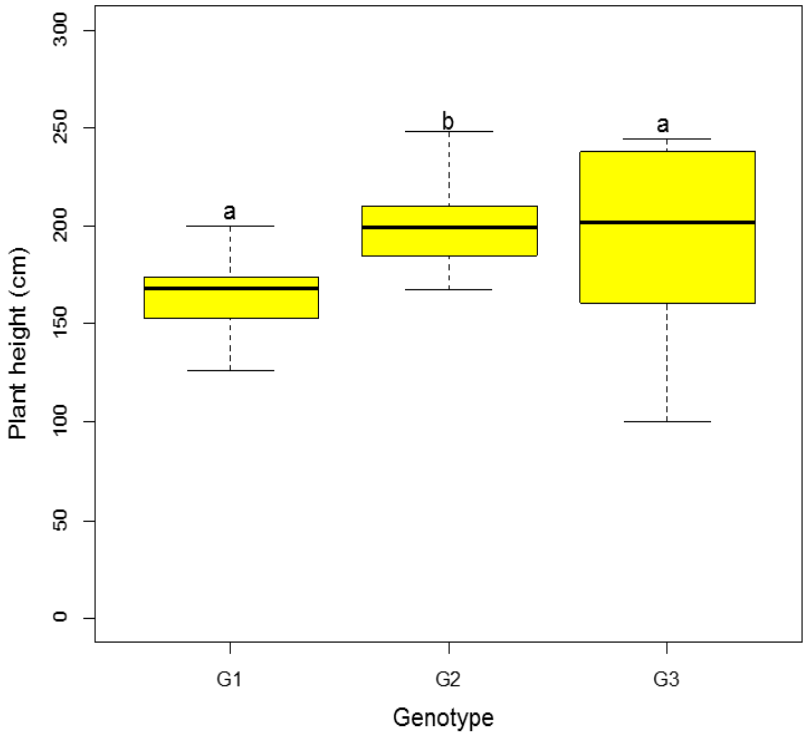


Figure 3.9 Genotypes in the H2 allohexaploid population derived from the cross *(B. napus* × *B. carinata)* × *B. juncea*, showing significant differences between genotypes in plant height, ($p < 0.05$, a and b Tukey's HSD)

Analysis in the H2 allohexaploid population (H1-006, H1-014, H1-015, H1-016, H1-020, H1-022, H1-023, H1-040, H1-044, H1-052, H1-058) found significant differences between progeny sets and the mean number of bivalents ($p = 0.000375$), total number of chromosomes ($p = 0.000678$), plant height ($p = 0.000004$), pollen fertility ($p = 0.00003$) and total seed set ($p = 0.011$), (one-way ANOVA; Table S2). Post-hoc analysis using Tukey's HSD test also found significant differences ($p < 0.05$) between mean chromosome numbers in 60% of sib lines within progeny sets in "G1" and "G2" genotypes and between 20% of sib lines within progeny sets in "G2" and "G3" genotypes (Figure 3.10).

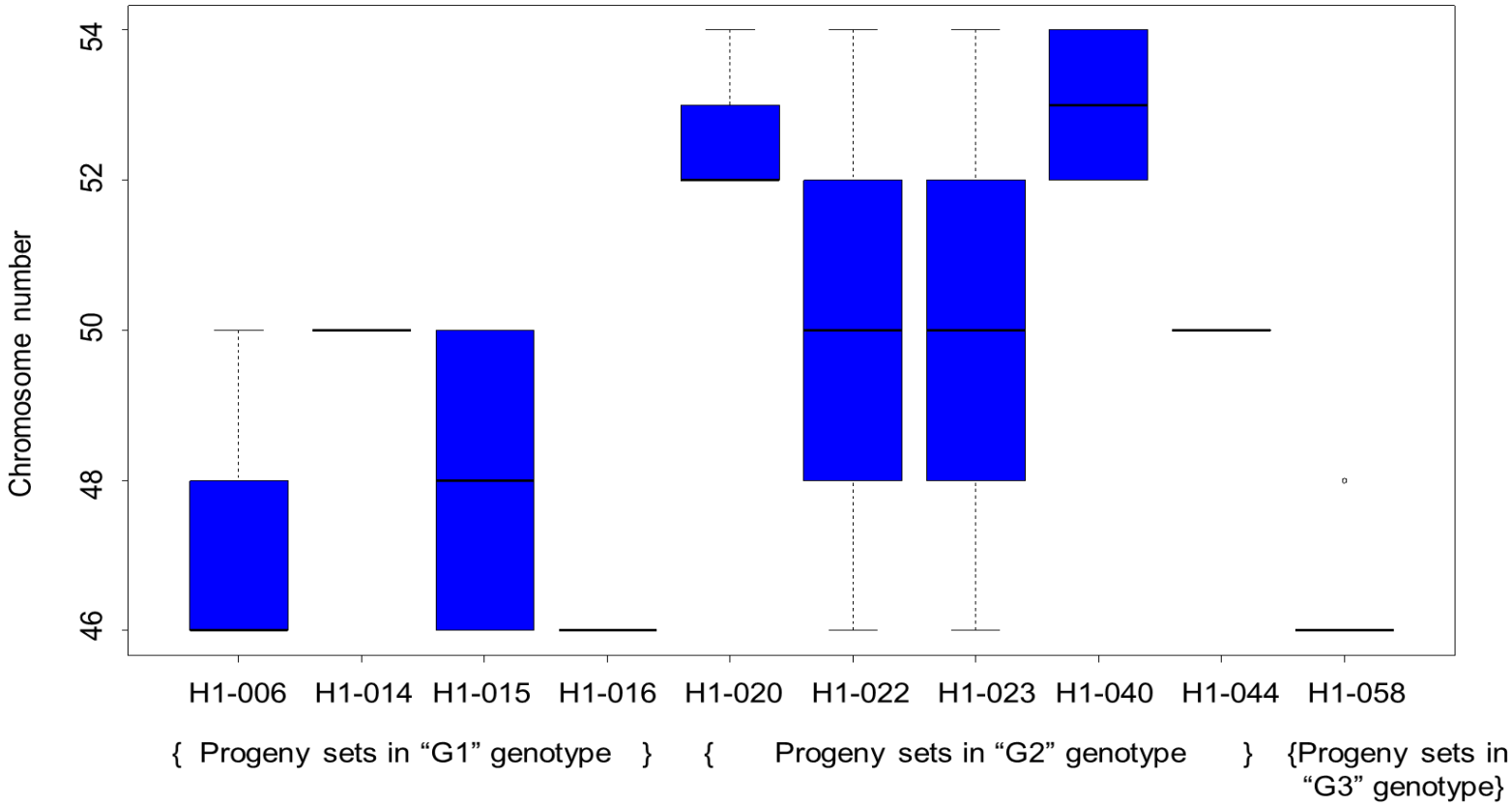


Figure 3.10 Progeny sets in the H2 allohexaploid population derived from the cross (*B. napus* × *B. carinata*) × *B. juncea*, showing significant differences in chromosome number, ($p < 0.05$, between progeny sets in “G1” and “G2” genotypes and between progeny sets in “G2” and “G3” genotypes, Tukey’s HSD)

Significant differences ($p < 0.05$) were found in the mean number of bivalents at metaphase 1 in 30% of sib lines within progeny sets in “G1” and “G2” genotypes and between 60% of sib lines within progeny sets in “G2” and “G3” genotypes (Figure 3.11). Additionally, significant differences ($p < 0.05$) were found in mean pollen fertility % between 20% of sib lines within progeny sets in “G1” and “G2” genotypes, between 16% of sib lines within progeny sets in “G2” and “G3” genotypes and between 33% of sib lines within progeny sets in “G2” genotype (Figure 3.12). Significant differences ($p < 0.05$) were also found in total seed numbers between 3% of sib lines within progeny sets in “G1” and “G2” genotypes, between 16% of sib lines within progeny sets in “G2” and “G3” genotypes and between 13% of sib lines within progeny sets in “G2” genotype (Figure 3.13). Finally, significant differences ($p <0.05$) were found in mean plant height in 40% of sib lines within progeny sets in “G1” and “G2” genotypes, between 66% of sib lines in progeny sets in “G1” and “G3” genotypes and between 50% of sib lines within progeny sets in “G2” and “G3” genotypes (Figure 3.14). Bonferroni correction for multiple testing was carried out at $\alpha = 0.05$: $p < 0.00278$.

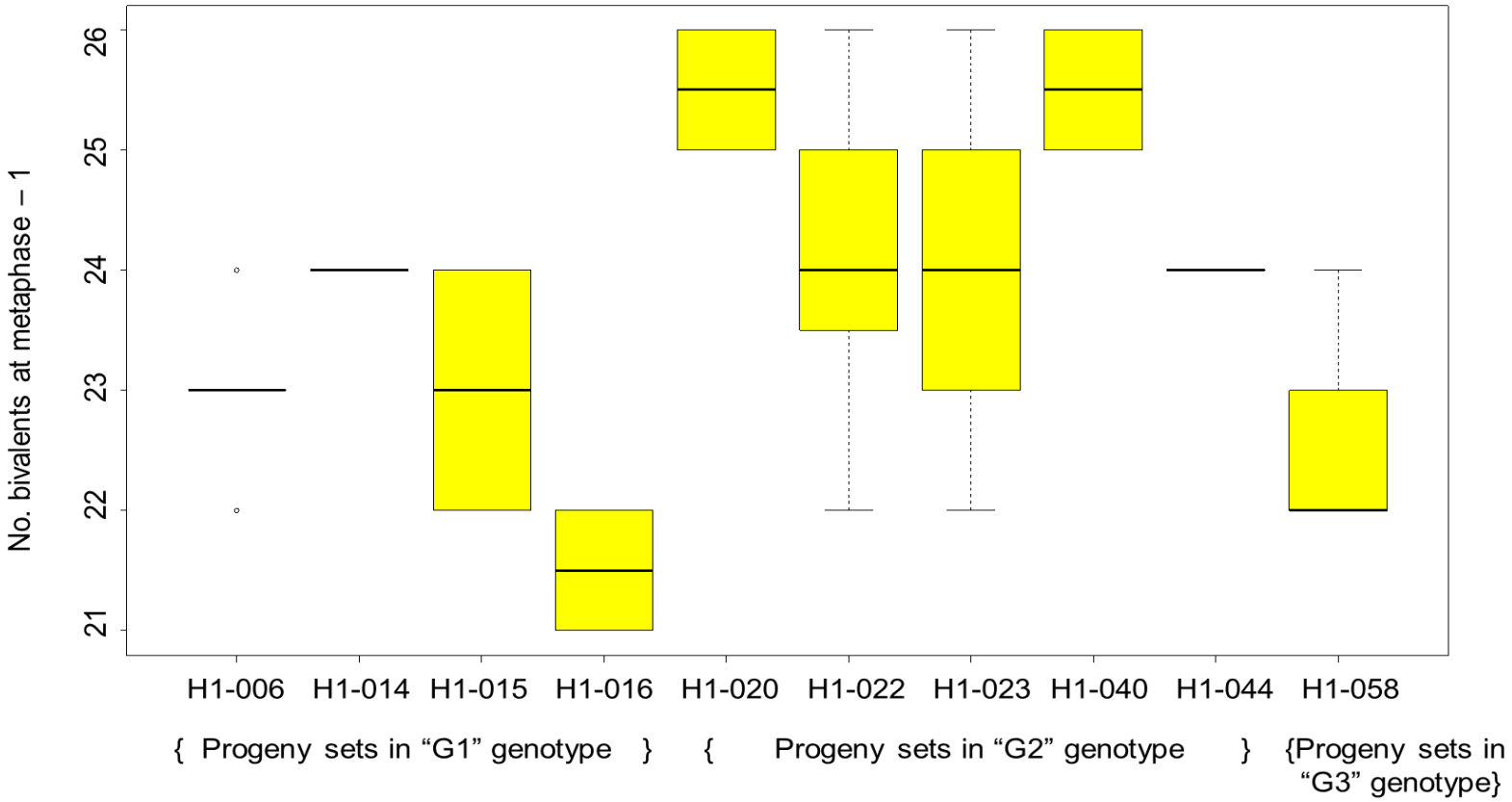


Figure 3.11 Progeny sets in the H2 allohexaploid population derived from the cross *(B. napus* × *B. carinata)* × *B. juncea*, showing significant differences in number of bivalents at metaphase 1 ($p < 0.05$, between progeny sets in “G1” and “G2” genotypes and between progeny sets in “G2” and “G3” genotypes, Tukey’s HSD)

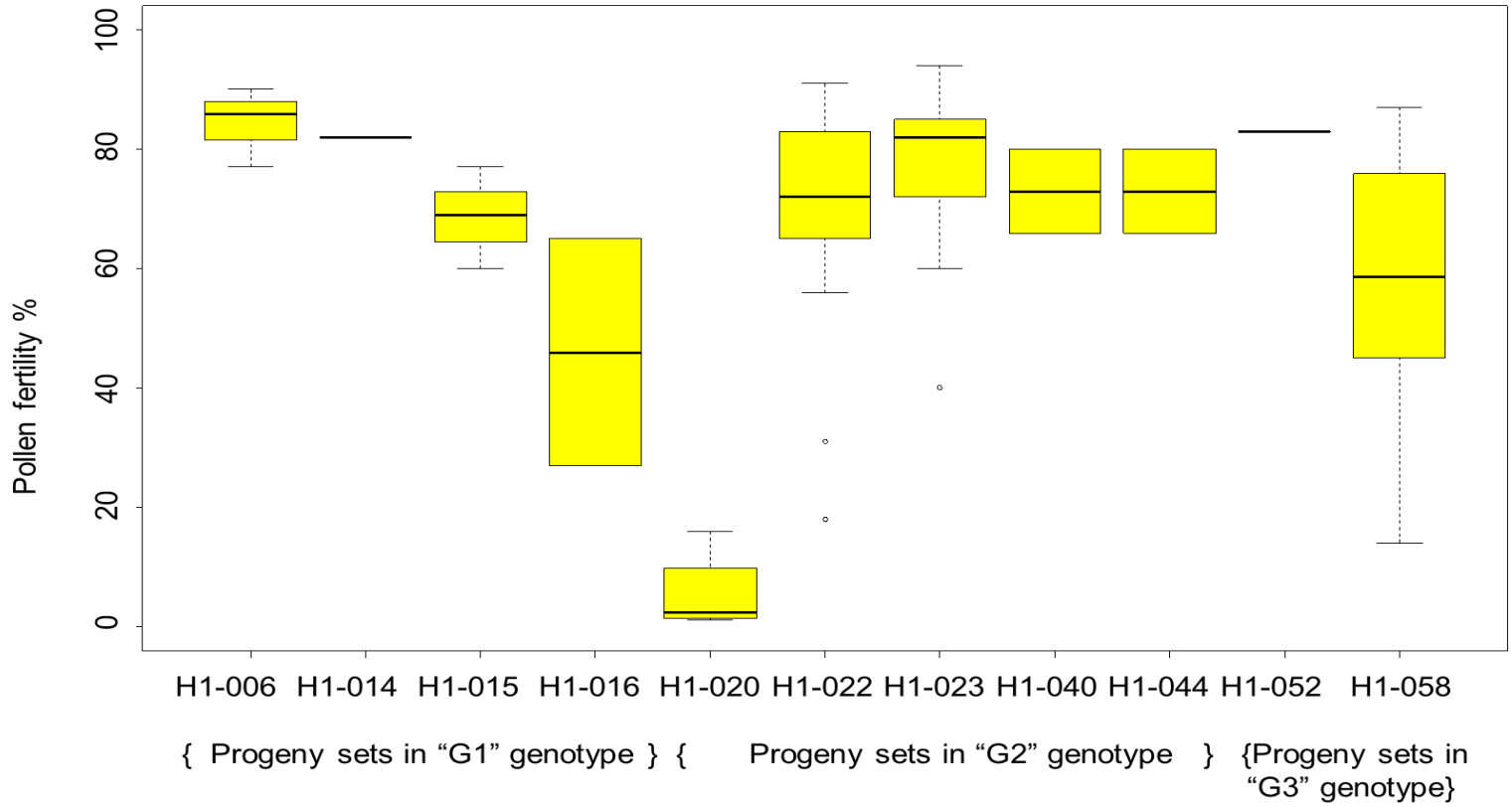


Figure 3.12 Progeny sets in the H2 allohexaploid population derived from the cross *(B. napus* × *B. carinata)* × *B. juncea*, showing significant differences in pollen fertility %, ($p < 0.05$, between progeny sets in “G1” and “G2”, between progeny sets in “G2” and “G3” genotypes and between progeny sets in “G2” genotype, Tukey’s HSD)

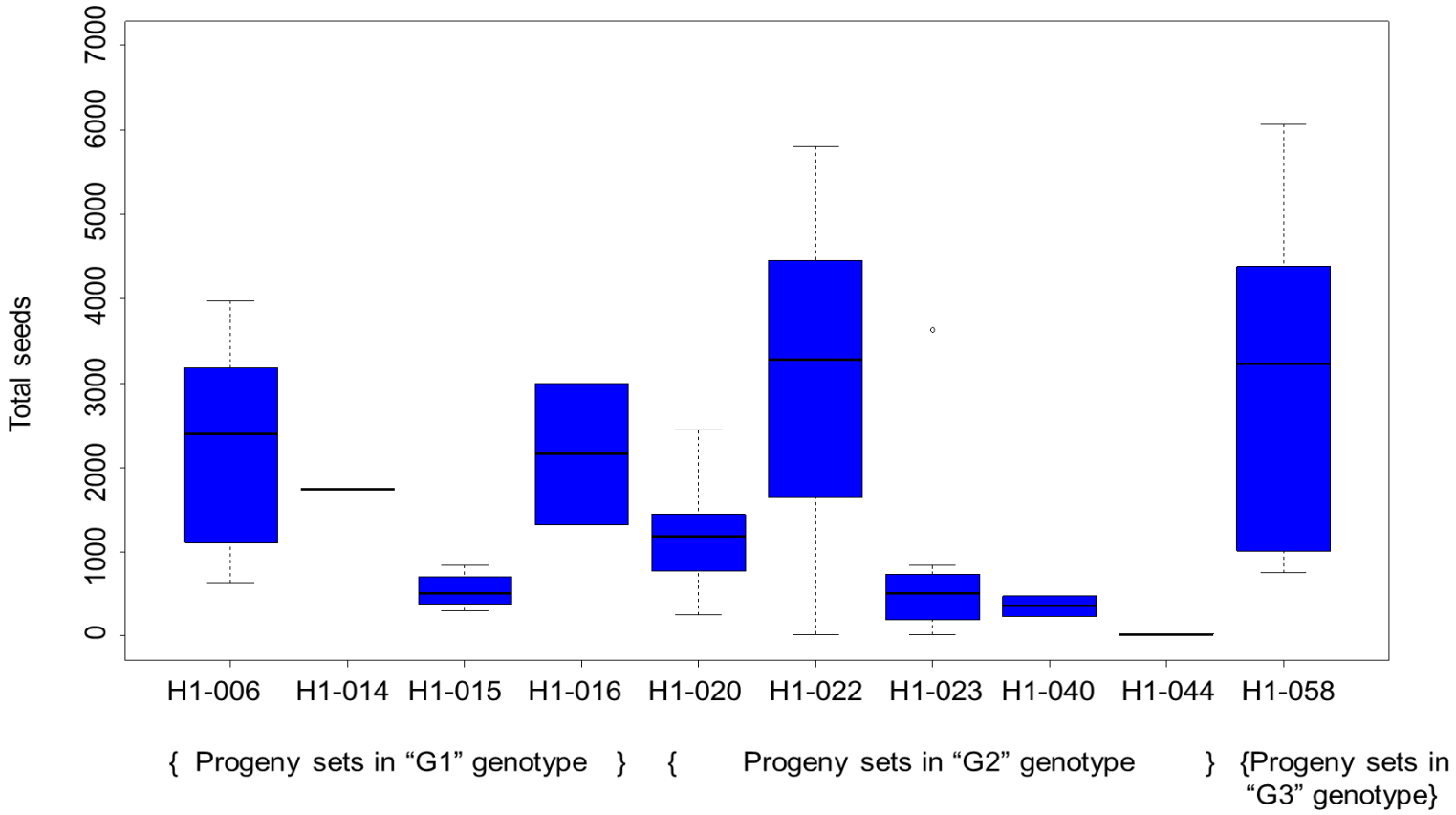


Figure 3.13 Progeny sets in the H2 allohexaploid population derived from the cross *(B. napus* × *B. carinata)* × *B. juncea,* showing significant differences in total seed set ($p < 0.05$, between progeny sets in “G1” and “G2” genotypes, between progeny sets in “G2” and “G3” genotypes and between progeny sets in “G2” genotype, Tukey’s HSD)

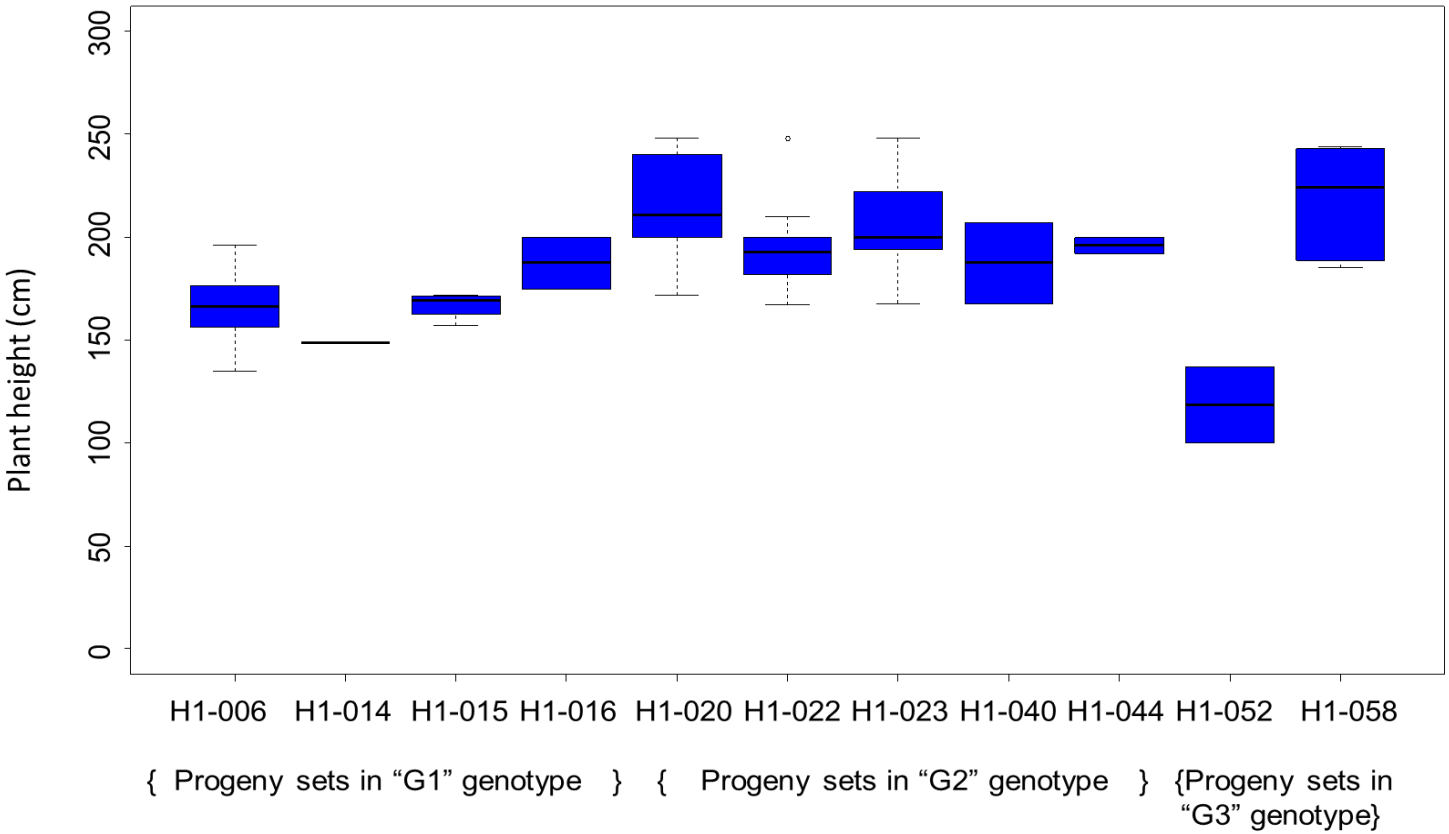


Figure 3.14 Progeny sets in the H2 allohexaploid population derived from the cross *(B. napus* × *B. carinata)* × *B. juncea*, showing significant differences in plant height, ($p < 0.05$, between progeny sets in “G1” and “G2” genotypes, between progeny sets in “G2”and “G3” genotype, and between progeny sets in “G1” and “G3” genotypes, Tukey’s HSD)

3.4 Discussion

The purpose of this study was to analyse fertility and meiotic stability in novel *Brassica* allohexaploids and to determine which factors were influencing these traits. Pollen fertility, chromosome behaviour and phenotypic traits were characterised in a homozygous allohexaploid A2 population and in a heterozygous H2 population, and the traits compared to control cultivar species. The control cultivars used in this study were commercial cultivars and different from the parent genotypes used to generate the A2 and H2 populations; controls were used to make inferences. Both the A2 and H2 populations had lower average pollen viability and seed set than the control plants. This may be attributed to the fact that the hexaploid populations are still segregating for fertility and meiotic stability. However, some individuals in these populations were within or in close range to the control samples for some traits. The number of seeds on the main shoot in the H2 was much lower than the control cultivar samples, which may possibly be attributed to the more "wild-type" branching habits of the *B. carinata* and *B. juncea* genotypes used to generate the H2 population. Average plant height in both the A2 and H2 populations was higher than in the respective controls' samples, suggestive of heterosis for growth traits.

In this study, the H2 population displayed a wide segregation range for fertility traits and an average meiotic configuration of 49 chromosomes. The H2 population was generated from heterozygous "F_1" parents with alleles from each of *B. juncea*, *B. napus* and *B. carinata* (approximately $A^jA^nB^j\,B^cC^{n/c}C^{n/c}$; Mason et al. 2012). Thus, we would expect variation for meiotic stability and fertility because of allelic segregation, because the initial hybrid was heterozygous. It is also known that natural *B. napus* must have some genetic factor/s preventing (most) non-homologous chromosome pairing that must be absent in most genotypes of *B. rapa* and *B. oleracea* as all synthetic *B. napus* identified to date is unstable (Song et al. 1995; Gaeta et al. 2007; Szadkowski et al. 2010, 2011). The same locus could act in allohexaploid *Brassica* that have *B. napus* parents to keep the A and C genomes from pairing.

Some variation was observed in the A2 population, but this may be due to non-homologous rearrangements, while variation in the H2 population could be due to either allelic variation or chromosome rearrangements. Low fertility and average chromosome numbers in the A2

population could be due to chromosome loss via laggards or non-homologous chromosome recombination events. In some previous studies a widespread preferential loss of C- genome chromosomes over A- genome chromosomes in allohexaploid lines was found (Ge et al. 2009; Zhou et al. 2016), while Mason et al. (2014) found two lines had an excess of C genome chromosomes and loss of A genome chromosomes, suggesting that the "preferential loss" of C genome chromosomes, as stated above, could be a selection pressure effect, where loss of the C genome is better tolerated than loss of the A genome in maintaining plant viability and fertility. Evidence shows that close homoeology between the A and C genomes promotes frequent homoeologous exchanges during meiosis, which are likely to lead to instability (Nicolas et al. 2007, 2012). Gaeta and Pires (2010) propose that homoeologous interactions in allopolyploids, such as resynthesized *B. napus*, can not only generate novel gene combinations and phenotypes, but also destabilize the karyotype leading to aberrant meiotic behaviour, reduced fertility and aneuploidy.

Brassica researchers have in the past created allohexaploids mainly to transfer useful traits from one species to another. An allohexaploid *Brassica* from a *B. carinata* × *B. rapa* cross with high fertility over a few generations was produced by Howard (1942), however, a later study with similar crosses found lower fertility in hybrids up to the fifth generation (Iwasa 1964). In more recent studies, Tian et al. (2010) produced similar *B. rapa* × *B. carinata* allohexaploids demonstrating an increased fertility and percentages of offspring with 2n = 54 chromosome complements up to the fourth generation using different genotype combinations, while Zhou et al. (2016) synthesised *Brassica* allohexaploids from different crosses, and after several generations found high fertility and stable breeding behaviour in allohexaploids from *B. rapa* × *B. carinata* and *B. juncea* × *B. oleracea*, and lower fertility in allohexaploids from newly combined diploid genomes. Improved fertility in the A2 population may be achieved by using different parental crosses from different genetic backgrounds as a means of improving *B. rapa* × *B. carinata* variation for meiotic stability alleles (Tian et al. 2010).

Statistical analysis showed significant differences in cytological traits, such as chromosome number and bivalent formation, between sib lines within progeny sets as well as between genotypes in the H2 population. This implies that parent chromosome and allele complement, as well as starting parent genotype, is affecting fertility and meiotic stability in the H2

population. Although it was not possible to distinguish which parental - genotype combinations contributed to these differences in this study, previous work suggests *B. napus* is likely to harbour allelic variation for meiosis traits. Cifuentes et al. (2010a) found that two different meiotic phenotypes in *B. napus* for the *PrBn* allele came from different parent *B. oleracea* genotypes. In related studies, Sheidai et al. (2006) found cytogenetic variability in canola *(B. napus*) cultivars to be genotype specific. Differences in meiotic behaviour were found between three AABC (*B. juncea* × *B. napus*) genotypes (Mason et al. 2010), while variation between CCAB (*B. napus* × *B. carinata*) hybrid genotypes was found for homoeologous and homologous recombination frequencies and A-B, B-C and A-C pairing (Mason et al. 2011).

To date, limited data exists on meiotic behaviour in *Brassica* allohexaploids. Further cytogenetic analysis is required in subsequent populations of allohexaploids to identify genotypes exhibiting stable meiotic characteristics. Coupling this meiotic phenotyping with genotyping analysis in future may allow identification of underlying genetic mechanisms involved in meiotic stability and bring researchers closer to making a stable allohexaploid *Brassica* for agricultural benefit.

Chapter 4: "Doubled-haploid" allohexaploid *Brassica* lines lose fertility and accumulate genetic variation due to genomic instability

This chapter was submitted to Chromosoma on 17th October 2018

4.1 Introduction

Interspecific hybridisation is a key factor in speciation through formation of fertile and genetically diverse species in agriculture (Abbott et al. 2013). Some example of interspecific crops that have occurred as result of hybridisation events include bread wheat (*Triticum aestivum*), sisal (*Agave sisalana*), coffee (*Coffea arabica*), banana (*Musa acuminata*), cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*), maize (*Zea mays*), sugarcane (*Saccharum spp.*) and oats (*Avena sativa)* (Leitch and Leitch 2008; Song et al. 2012). Newly formed species may benefit from increased allelic diversity, increased heterozygosity, novel phenotypic variation and an ability to easily adapt to new environmental niches (Comai 2005; Udall and Wendell 2006). However, many new interspecific hybrids do not initially have stable meiosis, which is revealed as non-homologous interactions between chromosomes from different sub genomes during meiosis, leading to chromosome loss, instability and infertility (Mason et al. 2015). In this regard, a sorting out process is important to produce balanced gametes with the aim of preventing multiple or illegitimate chiasmatic associations that would cause aneuploidy (Cifuentes et al. 2010b; Gaeta and Pires 2010).

The *Brassica* species interrelationships, as evident in U's Triangle (Morinaga 1934; U 1935) is an example of natural hybridisation events that have generated new species and contributed to differentiation within a genus (Branca and Cartea 2011). Although the combination of the A, B and C genomes in one *Brassica* species does not occur naturally, a new allohexaploid *Brassica* (2n = AABBCC) may have potential for increased hybrid vigour and adaptation (Chen et al. 2011). However, to date a meiotically stable allohexaploid *Brassica* does not existing agriculture (Chen et al. 2011; Mason and Batley 2015). Previous attempts to develop a stable allohexaploid *Brassica* have been largely unsuccessful due to aberrant meiosis and the occurrence of aneuploid plants in the selfed progenies (Gupta et al. 2016).

However, other studies on allohexaploids synthesised from *B. carinata* × *B. rapa* have shown an increase in meiotic stability as the generations advanced while the proportion of progeny with 54 chromosomes increased with successive self-pollination and selection in each generation (Tian et al. 2010; Zhou et al. 2016). In a different study, near-allohexaploid *Brassica* hybrid populations with variable fertility and chromosome inheritance were produced from the cross (*B. napus* × *B. carinata*) × *B. juncea* (Mason et al. 2012), while another study reported stable 2n = 54 chromosome lines over four generations resulting from a cross between *B. rapa* × *B. carinata* (Gupta et al. 2016).

The field of genomics continues to play a major role in improving agriculture productivity, including discovery of genetic variation which is critical in increasing performance and efficiency of plant breeding (Bevan et al. 2017). Genomic rearrangements due to homoeology between the A and C genomes are seen frequently in *Brassica* interspecific hybrids (Nicolas et al. 2007, 2012), where they can become an important source of genetic and phenotypic variation (Stein et al. 2017). The most commonly applied molecular tools for crop improvement are molecular genetic markers. Single nucleotide polymorphisms (SNPs) are genetic markers based on single nucleotide substitutions of one base for another in DNA sequences categorised as transversions, transitions, and insertions/deletions. SNPs remain the preferred choice for numerous research and breeding applications because of their high prevalence in the genome and potential for strong linkage to selected traits (Hayward et al. 2012; Huang et al. 2013; Dalton-Morgan et al. 2014). The *Brassica* 60K Illumina Infinium SNP array is a valuable tool for SNP genotyping due to the speed in attainment of data, as well as ease of analysis (Clarke et al. 2016; Mason et al. 2017).

Microspore culture is utilised widely in many crop species, including *Brassica* oilseeds, to generate haploid and doubled haploid (DH) homozygous lines and germplasm for breeding and genetic analysis (Xu et al. 2007; Cousin et al. 2009). Haploid plants produced from an F1 population combine the two parental genomes and have only one allele at every locus. Converting sterile haploids into fertile doubled haploids (DH) produces immortal homozygous lines which are useful in fixing traits quickly in desirable combinations in a variety (Seymour et al. 2012).

In this study, we hypothesised that all plants within a progeny set (MDL) in an allohexaploid *Brassica* would be genetically identical, and that any variation observed would be due to meiotic instability. As secondary hypotheses we proposed: a) that non-identical plants within an MDL are meiotically unstable and aimed to validate this by quantifying meiosis in 2-3 plants per individual MDL and (b) that non-homologous translocations in the MDL1 generation will result in meiotic instability and subsequent non-homologous translocation events in subsequent generations. SNP data was examined for translocations between A and C chromosomes.

4.2 Materials and methods

4.2.1 Experimental material

The experimental material comprised self-pollinated seeds produced by first generation microspore-derived plants from a parent near-allohexaploid plant derived from the cross (*B. napus* × *B. carinata*) × *B. juncea*. These lines were predicted to have unstable meiosis to varying degrees. The production of the parent near-allohexaploid is described in Mason et al. (2012), while the MDL1 population was produced and previously analysed in Mason et al. (2014) and Mason et al. (2015). Fourteen microspore-derived plants from this population produced more than ten seeds (12 – 198 seeds); these "MDL2" seeds comprised the experimental population. A total of 6 – 26 MDL2 seeds per MDL1 were sown, with 2 – 15 seeds per line sown at each of four different timepoints between March 2015 and July 2016, dictated by survival rates. Seeds from each of the parent genotypes *B. napus* "Surpass400_024DH*", B. carinat*a "195923.3.2_01DH" and *B. juncea* "JN9-04" were planted as controls. Germination and plant growth were carried out under temperature-controlled glasshouse conditions (20-22°C) at the University of Western Australia, Perth (Figure 4.1).

Figure 4.1 Double haploid MDL2 population in the glasshouse at the University of Western Australia (UWA)

4.2.2 Pollen fertility and seed set

Ripened floral buds were collected early in the morning. Pollen grains were assessed by squashing anthers in 1% acetocarmine (prepared as described in section 2.2) on glass microscope slides. Observations were carried out using a compound light microscope. Pollen grains that were plump and darkly stained were considered viable, whereas unstained and shrivelled pollen were considered non-viable. A total of 300 pollen grains were scored from at least three flowers from each of the 42 plants. Seed set was also determined at the end of the growing season, after enclosing racemes in pollination bags to encourage self-pollination.

4.2.3 Meiotic chromosome observations

Floral buds were collected during the early morning and fixed in Carnoy's II solution (ethanol: chloroform: acetic acid 6:3:1) (prepared as described in section 2.1) for up to 48 hours and subsequently stored in 70% ethanol at 4^{o} C (prepared as described in section 2.4).

Preparation of the anthers involved staining followed by squashing in a drop of 1% acetocarmine solution (prepared as described in section 2.2) on glass microscope slides. Observations of the pollen mother cells (PMCs) were carried out at metaphase I and II stages of meiosis using a ZEISS light microscope (Bright field phase) and images captured using the Axio Vision Imaging system (Release 4.8.1).

4.2.4 DNA extraction and SNP genotyping

The DNeasy® Plant Mini Kit (QIAGEN©) was used for DNA isolation according to the manufacturer's instructions. DNA quality and quantity assurance were determined using 1% agarose gel electrophoresis (1 × TAE buffer) and by comparison to a known size ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder) and through DNA Qubit quantification (prepared as described in sections 2.7, 2.8, 2.9, 2.10, 2.11 and 2.12). DNA samples were normalised before genotyping. Illumina Infinium *Brassica* 60K SNP array (Illumina Inc., San Diego, USA) genotyping was carried out according to the manufacturer's instructions.

4.2.5 Analysis of SNP genotyping data

SNP array data was visualised using Illumina Genome Studio software: http://support.illumina.com/array/array software/genome studio/downloads.html.
The filtering and cluster analysis of the SNP genotyping data was done in Genome Studio, using cluster files generated by Mason et al. (2015) for the MDL1 allohexaploid *Brassica* parent population. SNPs falling slightly out of the cluster patterns were manually adjusted. Output data was exported into Excel tables.

Chromosome segments were determined to be absent based on the SNP data if more than five continuous SNPs had no call (NC) scores within a single individual but were present in other individual plants within the progeny and/ or the parents.

4.2.6 Data analysis

ANOVA analysis, Tukey's Honest Significant Differences tests and boxplots were carried out using R version 3.2.2 (The R Foundation for Statistical Computing 2016). The (aov) and (summary) R commands were run to determine Analysis of Variance (ANOVA) for the means of meiotic characteristics, chromosome numbers, pollen fertility % and seed set.

Tukey's Honest Significant Differences test (Tukey HSD) was used to establish significant differences between progeny sets for the assessed traits. Hierarchical cluster analysis was run to validate the relationship between the MDL1 parent and the MDL2 progeny populations, using function "hclust" with a Euclidean distance matrix in R library "pvclust". Copy number variation plots for A and Genome SNP alleles were plotted based on Log R ratio and B Allele frequencies calculated in genome Studio and plotted in R (Mason et al. 2015).

4.3 Results

4.3.1 Germination, survival and fertility

A total of 250 experimental MDL2 seeds were planted from 14 different MDL1 parents, with 6 – 26 seeds planted per MDL. Of these 250 seeds, only 95 germinated (38%), and 75 survived to flowering (30%), with an attack of *Alternaria* blight in one sowing trial accounting for the majority of the difference between germination and survival rates overall. Finally, 42 plants from 6 lines which germinated at similar timepoints in disease-free environments were genotyped and characterised, of which three were eliminated on the basis of out-pollination based on the genotyping data to leave a total of 39 individuals in the final experimental population (9, 5, 10, 1, 10 and 4 plants from MDL07, MDL23, MDL28, MDL30, MDL60, and MDL64 respectively).

In each of the six lines, self-pollinated seed set was lower on average in the MDL2 progeny generation relative to their MDL1 parent generation; only a single MDL2 plant exceeded its MDL1 parent seed fertility (Figure 4.2). Estimated pollen viability was more variable, with MDL1 and MDL2 showing similar results for most progeny sets (Figure 4.3) with the exception of the most seed-fertile parent MDL07, which showed 73% pollen viability but whose MDL2 progeny showed only 5 – 25% pollen viability (average 15%).

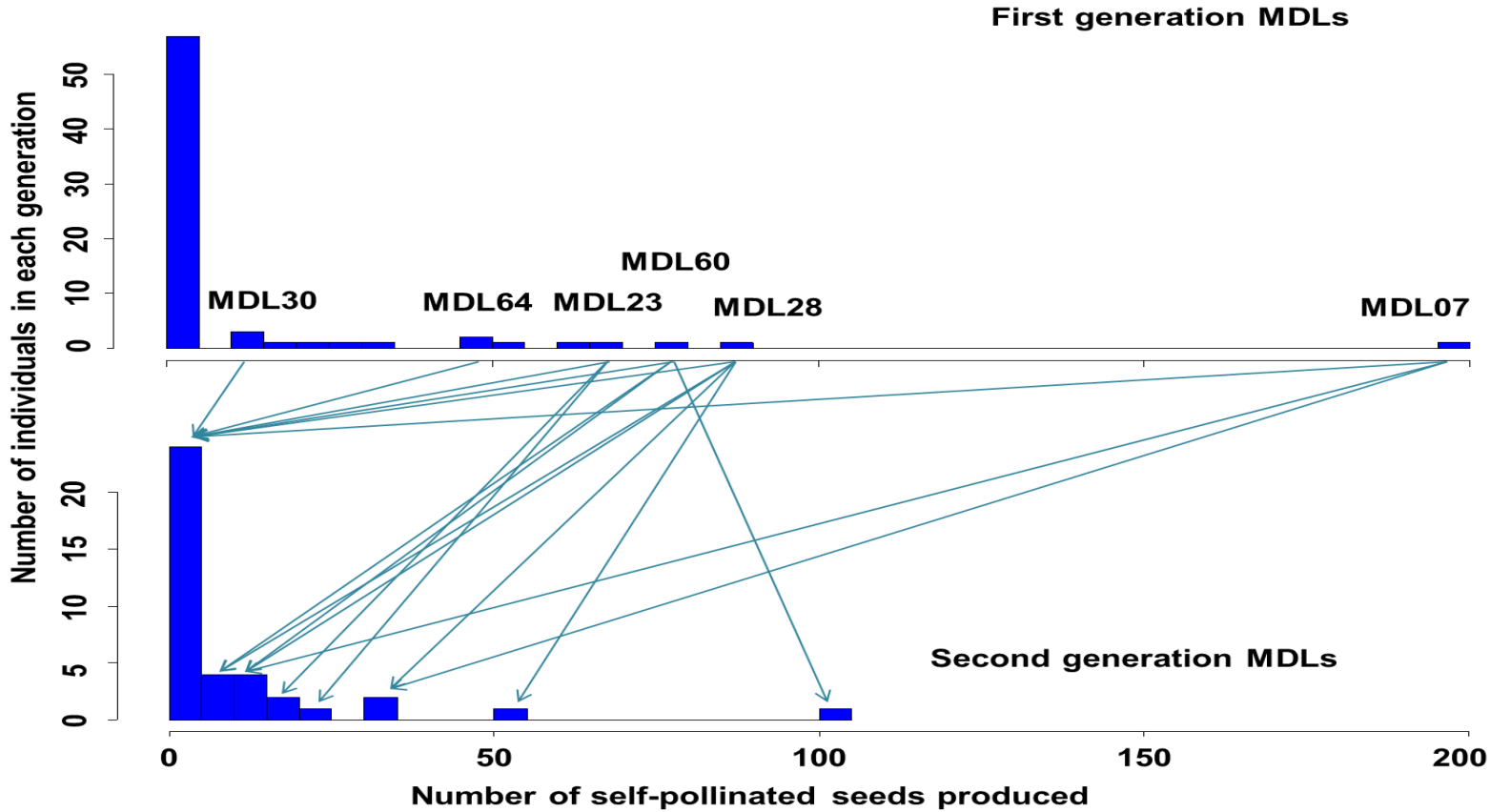


Figure 4.2: Self-pollinated seed production in the first (MDL1) and second (MDL2) generations of microspore-derived lines from a *Brassica* hybrid individual derived from the cross (*B. napus* × *B. carinata*) × *B. juncea*. Arrows indicate a parent/progeny relationship between MDL1 and MDL2 generations

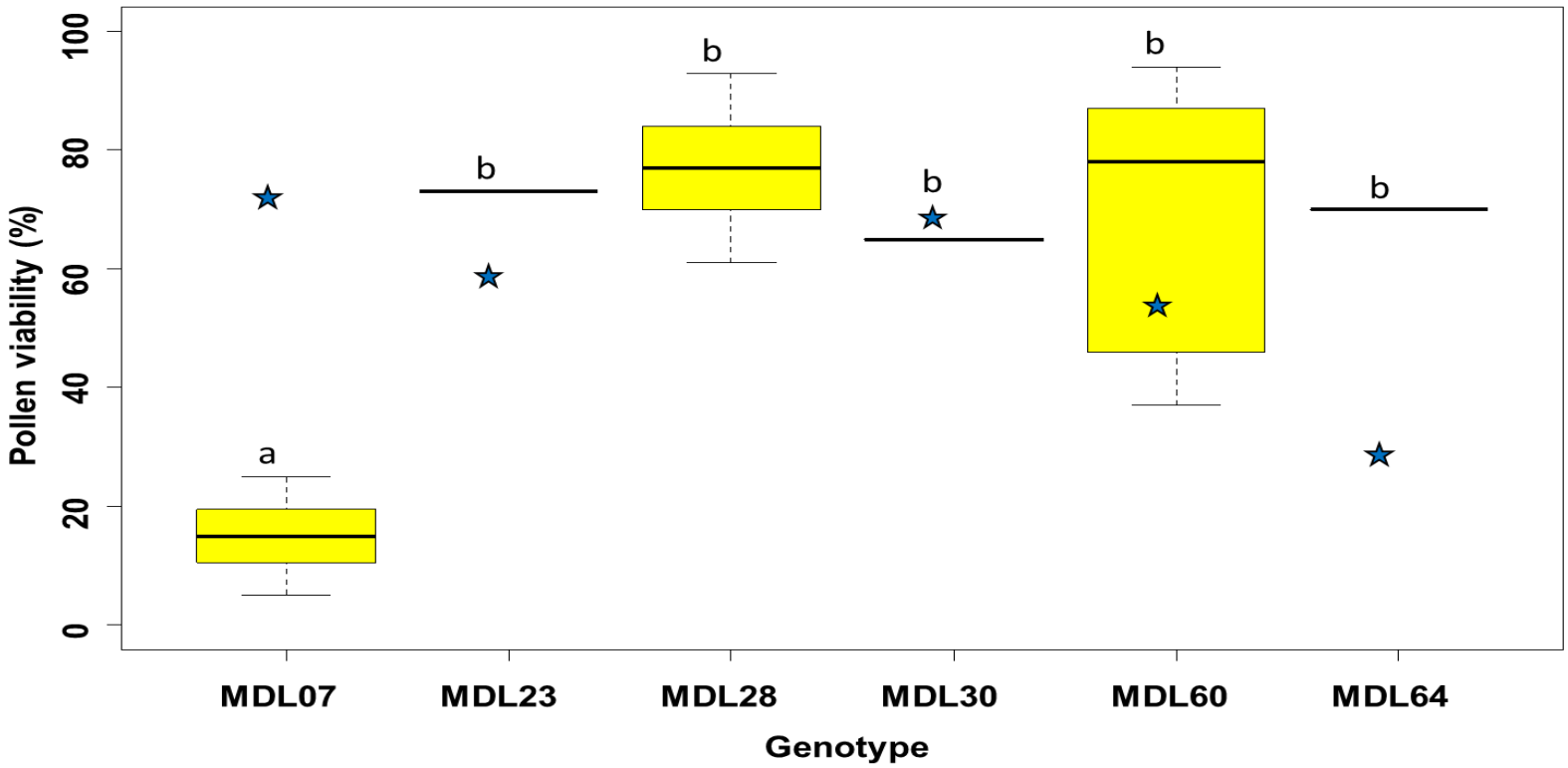


Figure 4.3: Pollen viability in second-generation individuals derived from microspores of a (*B. napus* × *B. carinata*) × *B. juncea* hybrid. First generation parent pollen viability is indicated with a blue star for each line

4.3.2 DNA quality and quantity check

DNA quality and quantity checks were carried out to determine the amount of DNA obtained and to determine the concentration and level degradation if any. Concentration readings, taken using a QUBIT 3.0 Fluorometer (Life Technologies), showed good quality DNA. DNA samples were diluted and optimised for DNA SNP chip array analysis (Figure 4.4).

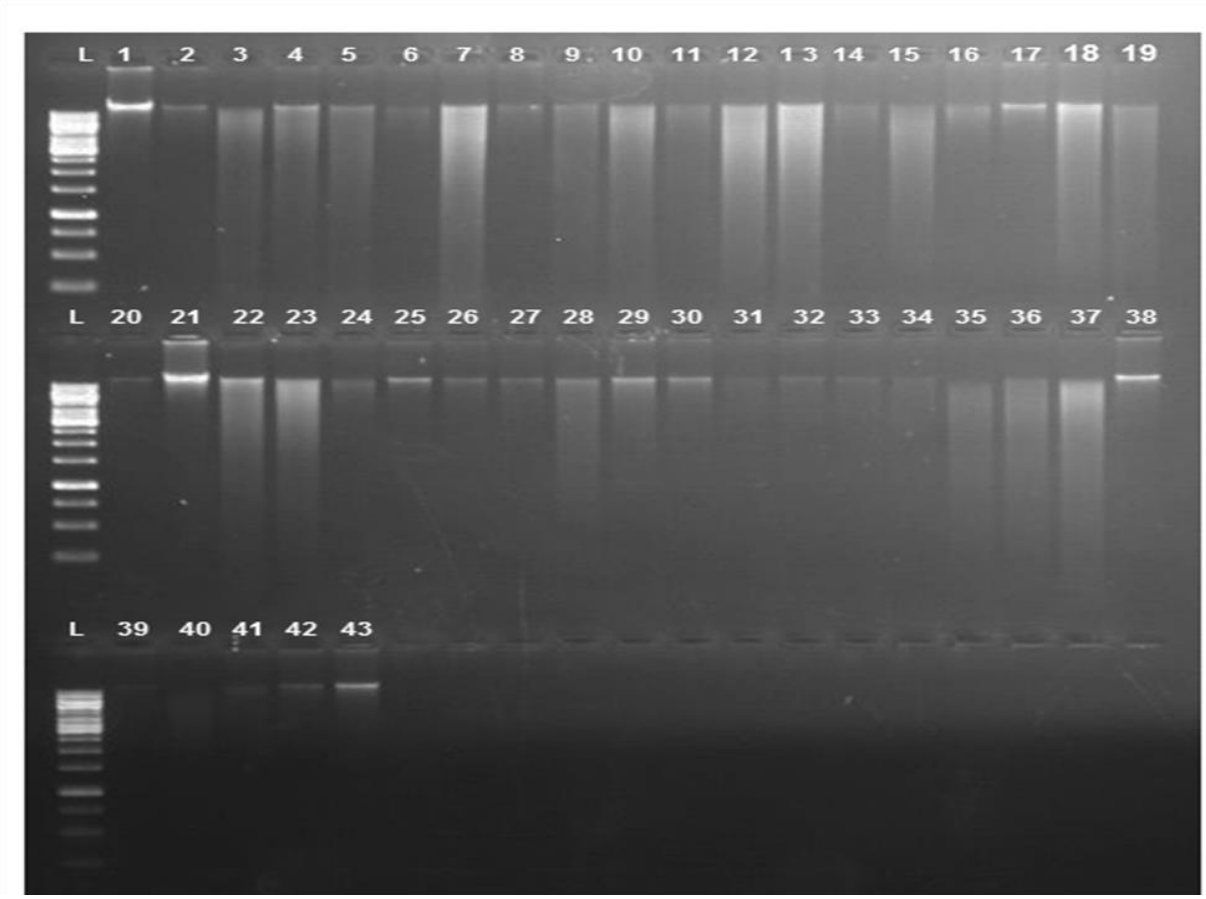


Figure 4.4 Gel electrophoresis image of MDL2 population: DNA samples 1 – 43 are MDLs 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8. 7.9, 7.10, 28.1, 28.2, 28.3, 28.4, 28.5, 28.6, 28.7, 28.8, 28.9, 28.10, 60.1, 60.2, 60.3, 60.4, 60.5, 60.6, 60.7, 60.8, 60.9, 60.10, 23.1, 23.2, 23.3, 23.4, 23.5, 23.6, 23.7, 64.1, 64.2 64.3, 31.2, 31.2, *B. napus* and L; 1 Kb ladder

4.3.3 Variation between microspore-derived lines in fertility and meiotic behaviour

MDL2 progeny sets differed significantly in pollen fertility ($p < 0.0001$, one-way ANOVA) but not in self-pollinated seed set ($p > 0.1$, one-way ANOVA). Significant differences were observed between the number of univalents (unpaired chromosomes) at metaphase I of meiosis ($p = 1.54e\text{-}15$ ***) and total number of chromosomes present ($p = 0.00185$ **) in MDL 2 progeny sets. Significant differences ($p < 0.05$) were only observed for the number of univalents and total chromosomes between MDL60 and MDL28 (Figure 4.5A, 4.5B) within a confidence level of 95% (Tukey's HSD, $p < 0.05$).

4.3.4 Evidence of meiotic instability in MDLs

Meiotic behaviour was assessed for progeny sets MDL28 and MDL60 but could not be determined in MDL7, MDL23 and MDL64 due to inability to obtain clear, good quality meiotic slides. The minimum number of pollen mother cells (PMCs) counted was ten cells, maximum was 35 cells, while the average was 20 cells. Plants MDL28_7, MDL28_8 and MDL 28_9 comprised aneuploid and unstable progeny: MDL28_7 had a range of 2 - 20 univalents (mode 20), a range of 22 - 24 bivalents (mode 24) with total chromosomes 40 – 48 (mode 48) at both metaphase I and II. MDL 28_8 had 2 univalents, a range of 20 – 24 bivalents (mode 20) and 40 – 48 total chromosomes (mode 40) at metaphase I MDL 28_9 had 20 univalents and a total of 40 chromosomes at metaphase II (Figure 4.6A). Variation was also observed between MDL60 progeny: MDL60_3 had a range of 20 - 22 bivalents (mode 20) and a range of 40 - 44 total chromosomes (mode 40) at metaphase I. MDL60_5 was characterised by up to 2 - 3 univalent, a range of 22 - 24 bivalents (mode 24), and 44 – 48 total chromosomes (mode 48) at metaphase II. While MDL 60_6 had 3 - 5 univalents, a range of 21 – 24 bivalents (mode 24) and 45 – 49 total chromosomes (mode 48) at metaphase II, some multivalents observed (Figure 4.6B).

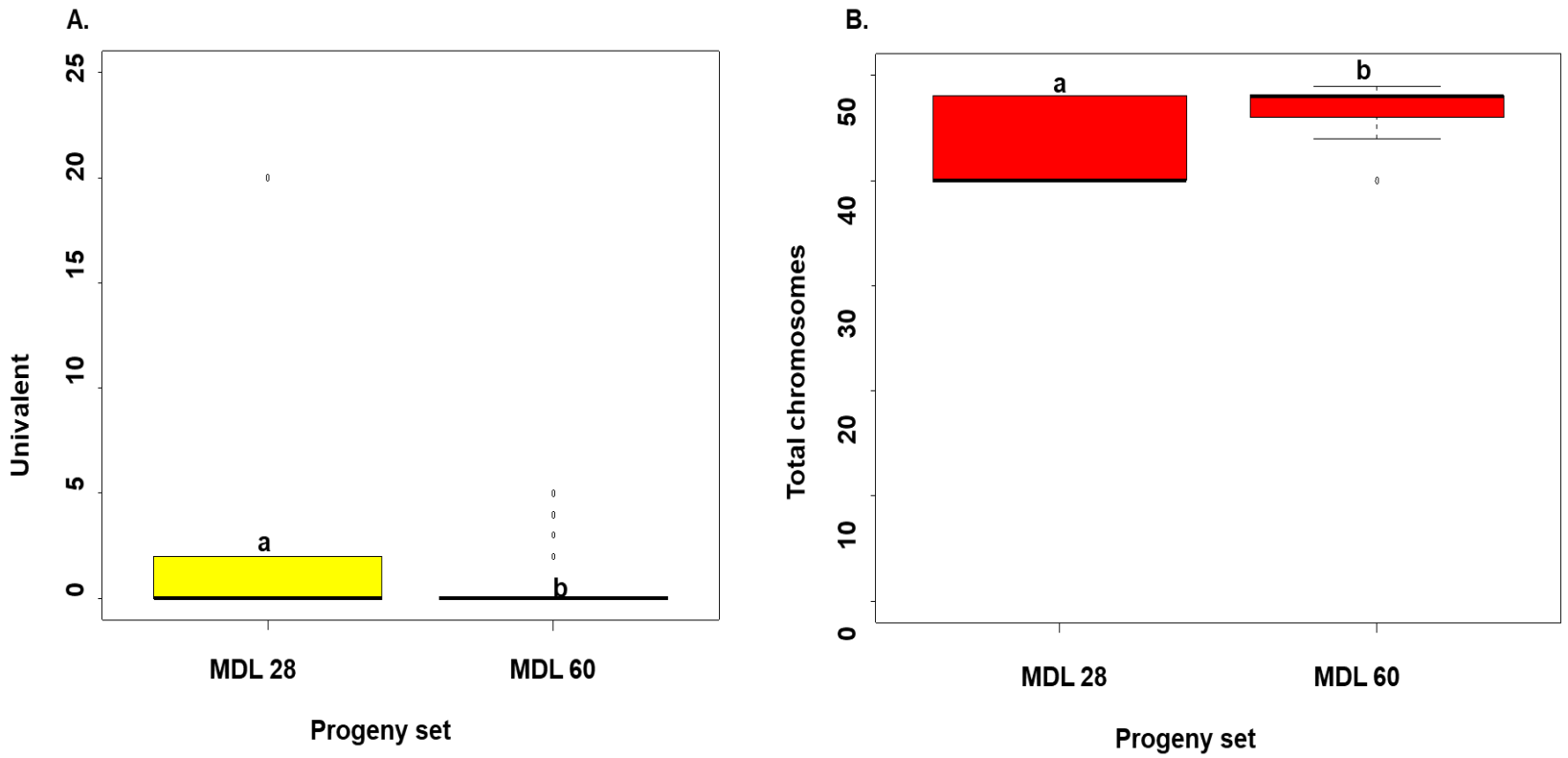


Figure 4.5 Number of univalent at metaphase I of meiosis and total chromosome numbers in progeny sets MDL 28, and 60 of the MDL2 population (self-pollinated progeny from MDL1 individuals, which were derived from microspores of a (*B. napus* × *B. carinata)* × *B. juncea* allohexaploid hybrid

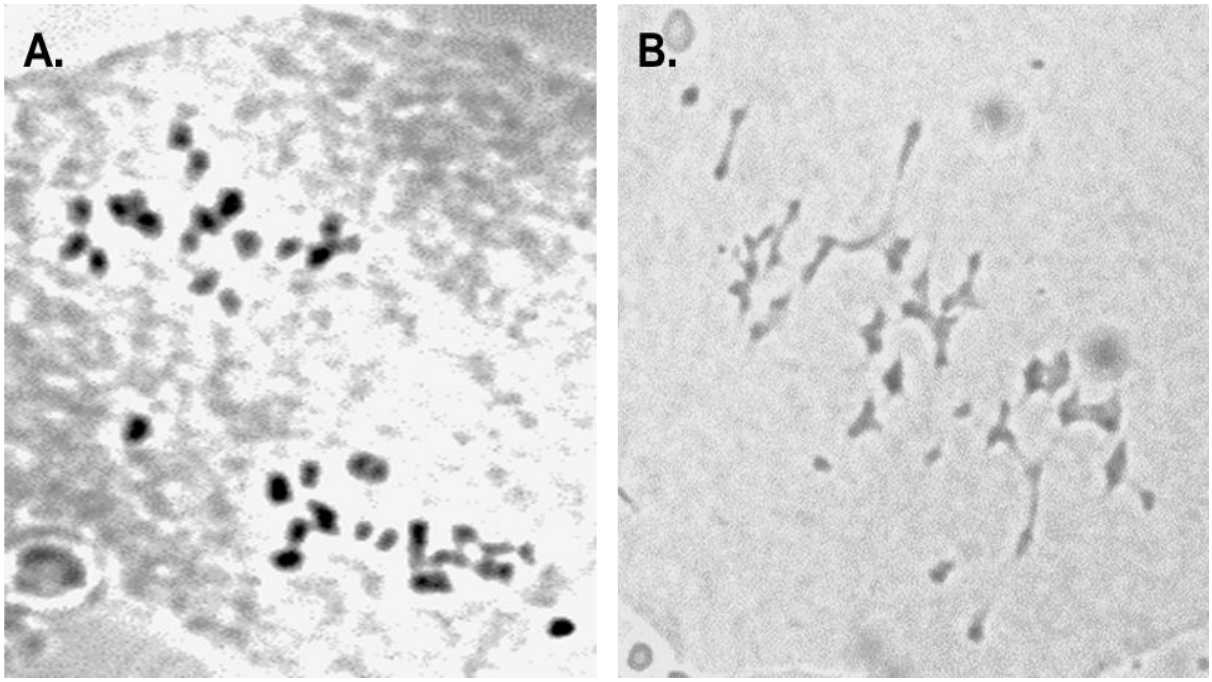


Figure 4.6 Meiotic images from the MDL2 population: A: MDL 28_9 at metaphase II with 20I, 20I showing a putatively lagging univalent chromosome at metaphase II B: MDL 60_6 at metaphase 1 with 22II and 4I (Magnification 100 × objective lens)

4.3.5 Genetic variation observed within microspore-derived lines

A total of 7,651 high quality, single locus polymorphic markers were selected from a total of 52,157 SNP markers present on the *Brassica* 60K Infinium Illumina array: the same set as used in Mason et al. (2015) for these genotypes. Apparent heterozygosity at individual SNP loci in the parent MDL1 was observed due to homoeologous pairing between the A and C genomes, which resulted in duplication of some genome regions such that one allele was present at a different genomic locus in the doubled haploid population (Mason et al. 2015). In this study on the progeny MDL2 population, we sought to determine if a) these homeologous exchanges were now fixed in the population (i.e. not segregating in the MDL2) and b) if these translocations resulted in further non-homologous chromosome interactions. A total of 18 duplicated regions and 36 deleted regions were observed in the six MDL1 individuals. Of these, seven duplications and five deletions were fixed and appeared to be inherited stably from the MDL1 to the MDL2 generation within each microspore-derived line, while four novel deletions were seen. Additionally, entire chromosomes A02 and A09 were seen to missing because of loss during the initial hybridisation event (Mason et al. 2015).

Microspore-derived lines (containing doubled haploid individuals) are not expected to show any genetic variation either between individuals within a line, or from one generation to the next within a line. Most MDLs showed little variation from the MDL1 to MDL2 generations based on R generated SNP hierarchical cluster analysis (Figure 4.7). In this study we hypothesised that all lines within an MDL were identical, and indeed we found that all lines within the progeny sets in MDL7 and MDL23 were identical to each other and to their parents. However, this was not the case for lines found in the progeny sets MDL28, MDL60 and MDL64, where genetic non-identity was evident.

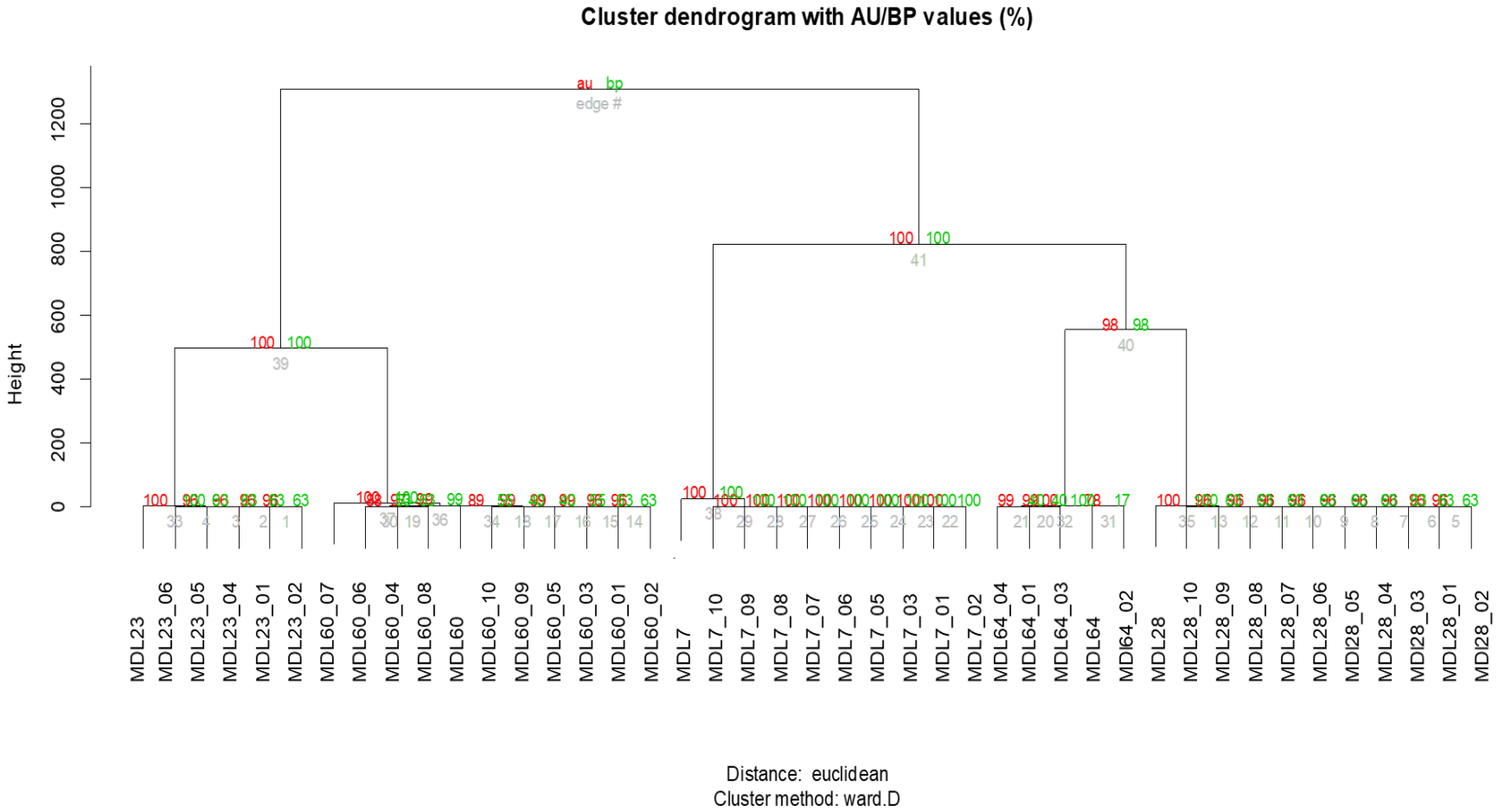


Figure 4.7 Cluster dendrogram showing the relationship between the parent MDL1 population individuals derived from microspores of a (*B. napus* × *B. carinata)* × *B. juncea* allohexaploid hybrid

The MDL23 progeny set contained duplications in chromosomes C05 (2.8 Mbp) and C09 (18.1 Mbp). This corresponded to deletions in homeologous regions (Chalhoub et al. 2014) on chromosomes A05 (0.7 Mbp) and A09 (11.3 Mbp) in both parent and progeny, while a duplication/deletion event for chromosome A07/C06 was also present. The MDL60 progeny set had a duplication in chromosome A03 (7.8 Mbp) that corresponded to a deletion in homeologous chromosome C03 (10.7 Mbp) in both parent and progeny. Other duplications in progeny MDL60 were in the chromosomes A10 (0.6 Mbp) and C04 (0.37 Mbp) but there were no corresponding deletions.

There was segregation observed on the translocated region from parent to progeny in 15% of chromosome A03 (5.35 Mbp) in the line MDL60_07, no other segregation was noted any other lines in the MDL60 nor in the other five progeny sets assessed. In the MDL64 progeny set there was a duplication in chromosome C01 (29.3 Mbp) that corresponded to a deletion in homoeologous chromosome A01 (15.5 Mbp) in both the parent and progeny. In the MDL28 progeny set there was a duplication in chromosome A01 (1 Mbp) that corresponded to a deletion in homoeologous chromosome C01 (3.2 Mbp) (Chalhoub et al. 2014).

Novel deletions (absent in MDL1 parent) were seen in MDL2 progeny in 1.7% of the stably inherited heterozygous region of chromosome A01 (0.4 Mbp), also in the lines MDL 28_6, MDL 28_7, MDL 28_8, MDL 28_9 and MDL 28_10. Additionally, they were also seen in 6.1% of chromosome C01 (2.6 Mbp) in the MDL60_3, in 92% of chromosome A09 (34.8 Mbp) in MDL60_4 and in 33% of chromosome C03 (22.1Mbp) in MDL60_7 (Table 4.1 and Supplementary Table1).

Additionally, some CNV plots based on fluorescence allele ratios revealed extra SNP clusters of AAB and BBA allelic ratios, indicative of three instead of two chromosomes in MDL 60_1 for chromosome A03 and in MDL64_3 for chromosome C02 (Figure 4.8 – 4.12).

Table 4.1 Inheritance of genomic structural variation between MDL1 individuals (derived from microspores of a (*B. napus* × *B. carinata)* × *B. juncea* allohexaploid hybrid) and their self-pollinated (MDL2) progeny

Type of genomic structural variation	MDL	Chromosome	Size in Mbp of variation	Chromosome length Mbp**	Unstable transmission to MDL2?
Duplication	MDL23	C05	2.8	46.8	No
Duplication	MDL23	C09	18.1	52.9	No
Duplication	MDL23	A07/C06*	41.9	66.5	No
Duplication	MDL28	A01	1.0	25.9	No
Duplication	MDL60	A03	7.8	35.7	Yes
Duplication	MDL60	A10	0.6	19.6	No
Duplication	MDL60	C04	0.37	53.3	No
Duplication	MDL64	C01	29.3	43.2	No
Deletion	MDL28_6,28_7,28_8, 28_9,28_10	A01	0.4	25.9	_
Deletion	MDL28,23,60,64	A02	26.4	26.4	_
Deletion	MDL23	A05	0.7	26.0	_
Deletion	MDL23	A09	11.3	37.9	_
Deletion	MDL23	A07/C06*	49.8	66.5	_
Deletion	MDL28	A09	37.9	37.9	_
Deletion	MDL28	C01	3.2	43.2	_
Deletion	MDL60_4	A09	34.8	37.9	_
Deletion	MDL60_3	C01	2.6	43.2	_
Deletion	MDL60	C03	10.7	67.0	_
Deletion	MDL60_7	C03	22.1	67.0	_
Deletion	MDL64	A01	15.5	25.9	_

* Pre-existing translocation between parent lines of *B. juncea*, *B. carinata* and *B. napus* used to produce the first-generation allohexaploid

** Chromosome length reference Chalboub et al. (2014)

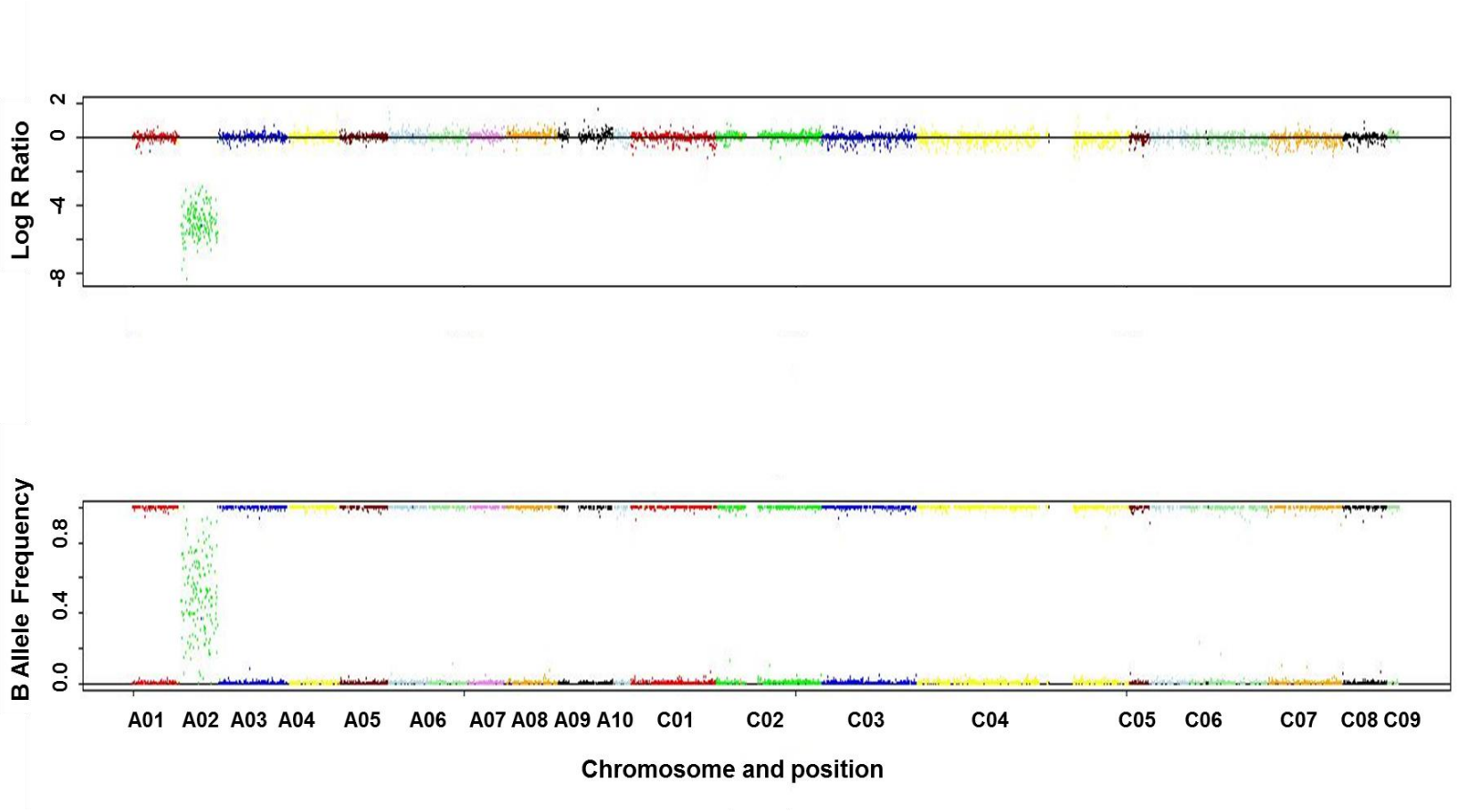


Figure 4.8 Copy Number Variation plots of MDL7_1 based on relative allele fluorescence ratios from Illumina Infinium *Brassica* 60K array SNP genotyping in MDL2 population shows a deletion in chromosome A02

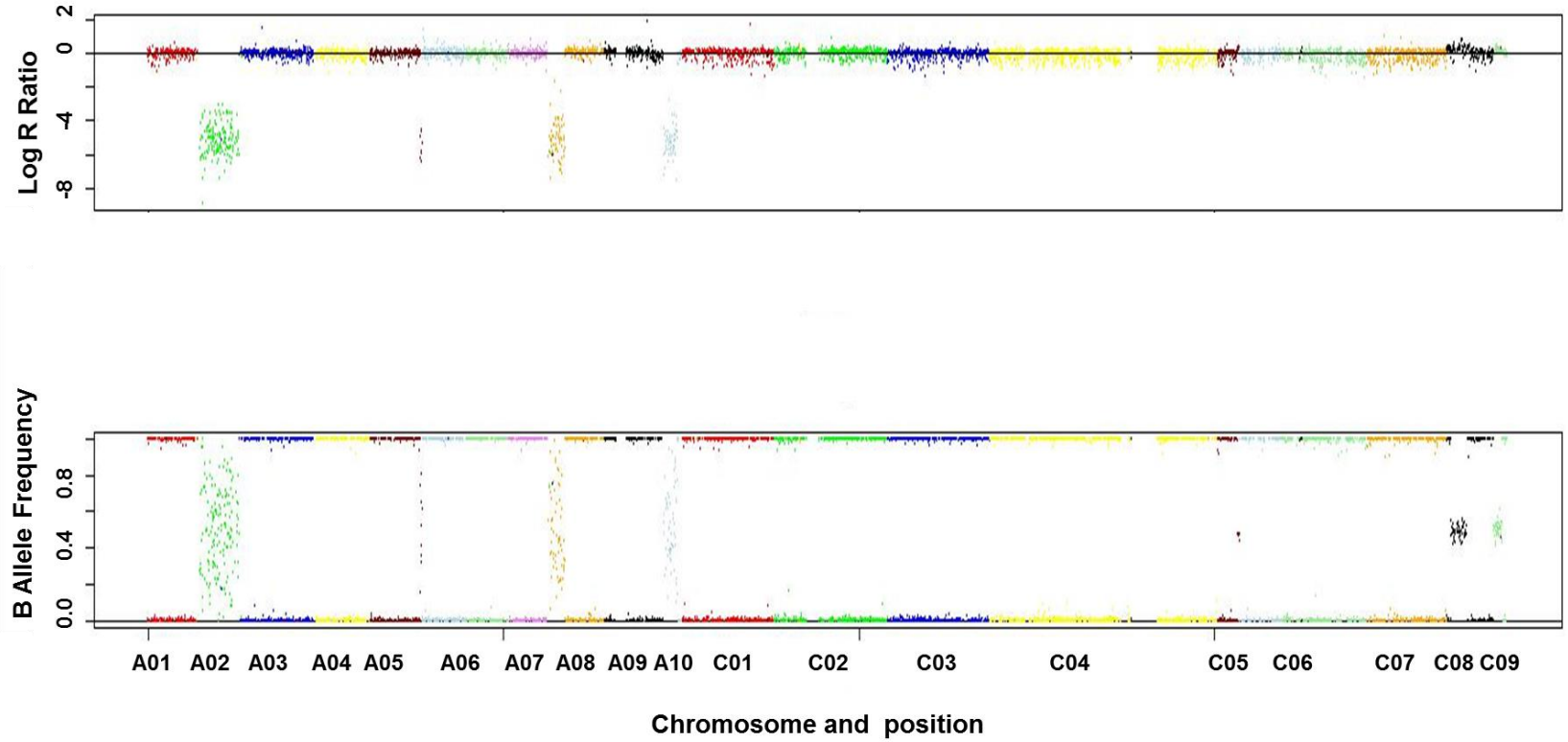


Figure 4.9 Copy Number Variation plots of MDL23_5 based on relative allele fluorescence ratios from Illumina Infinium *Brassica* 60K array SNP genotyping in MDL2 population shows deletion in chromosome A02, A07, A09 and duplication in C05 and C09

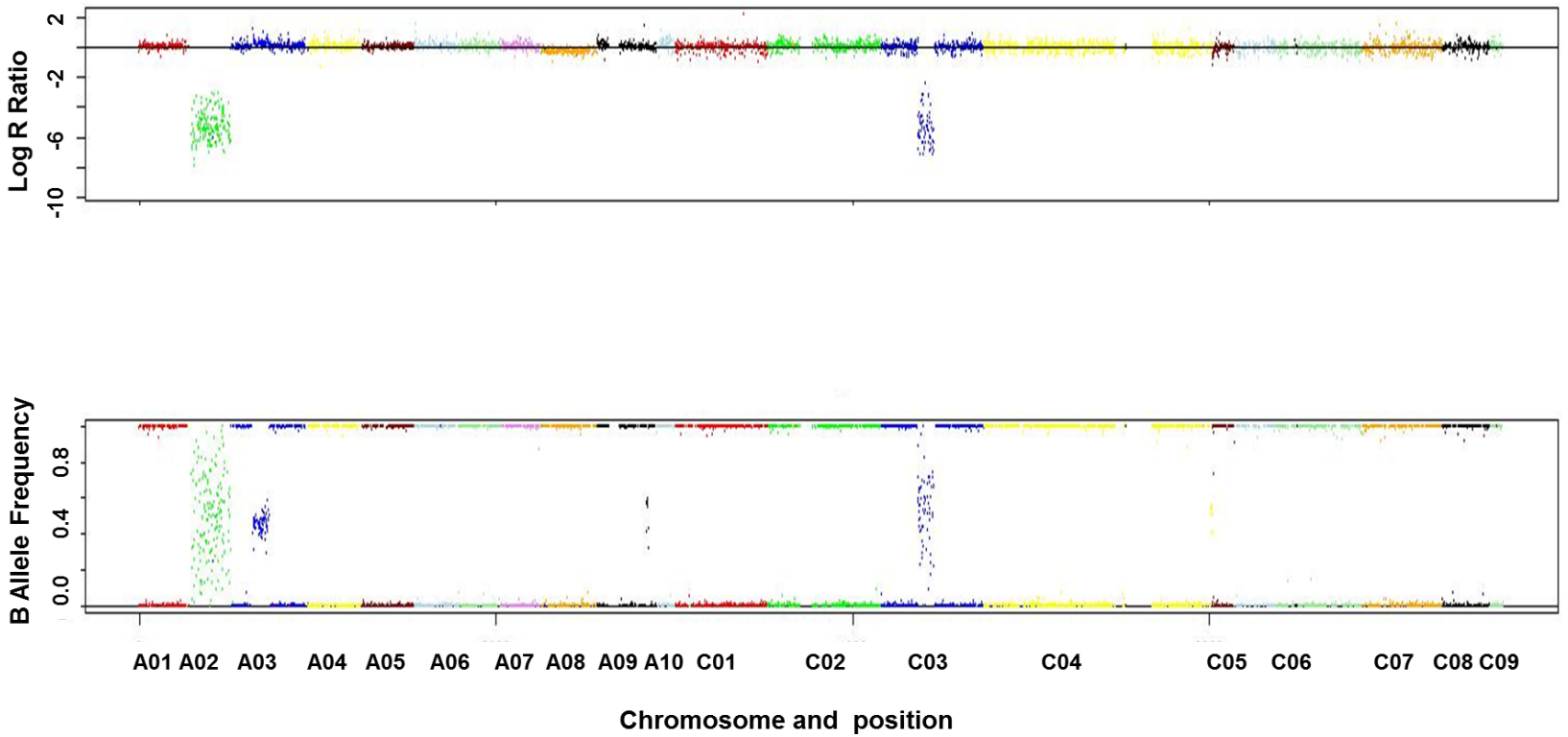


Figure 4.10 Copy Number Variation plots of MDL60_1 based on relative allele fluorescence ratios from Illumina Infinium *Brassica* 60K array SNP genotyping in MDL2 population shows a deletion in A02 and C03, a duplication in A03 and two chromosome clusters AAB and BBA in A03

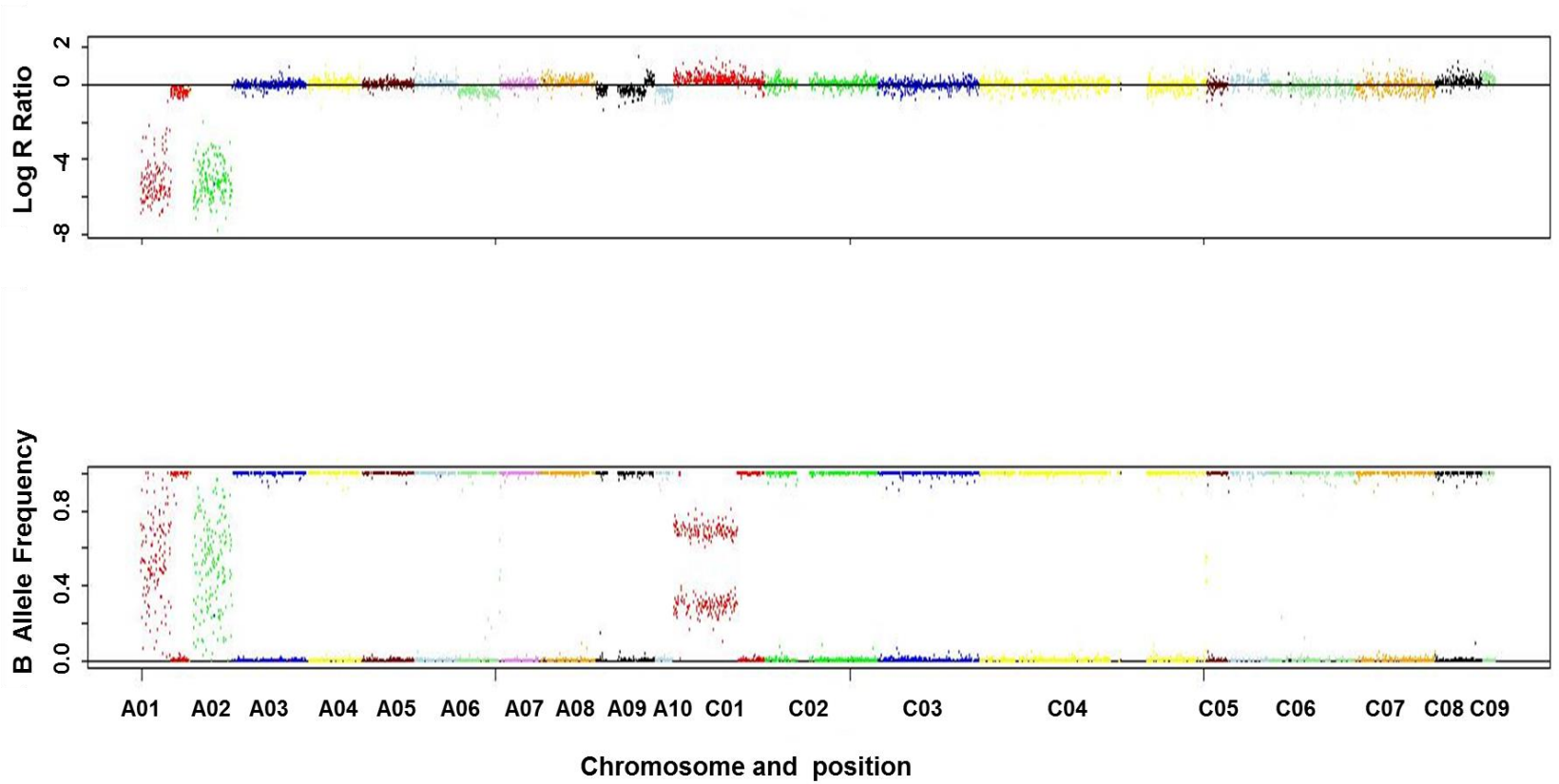


Figure 4.11 Copy Number Variation plots of MDL64_3 based on relative allele fluorescence ratios from Illumina Infinium *Brassica* 60K array SNP genotyping in MDL2 population shows a deletion in A01, A02 and a duplication in C01 and two chromosome clusters AAB and BBA in C02

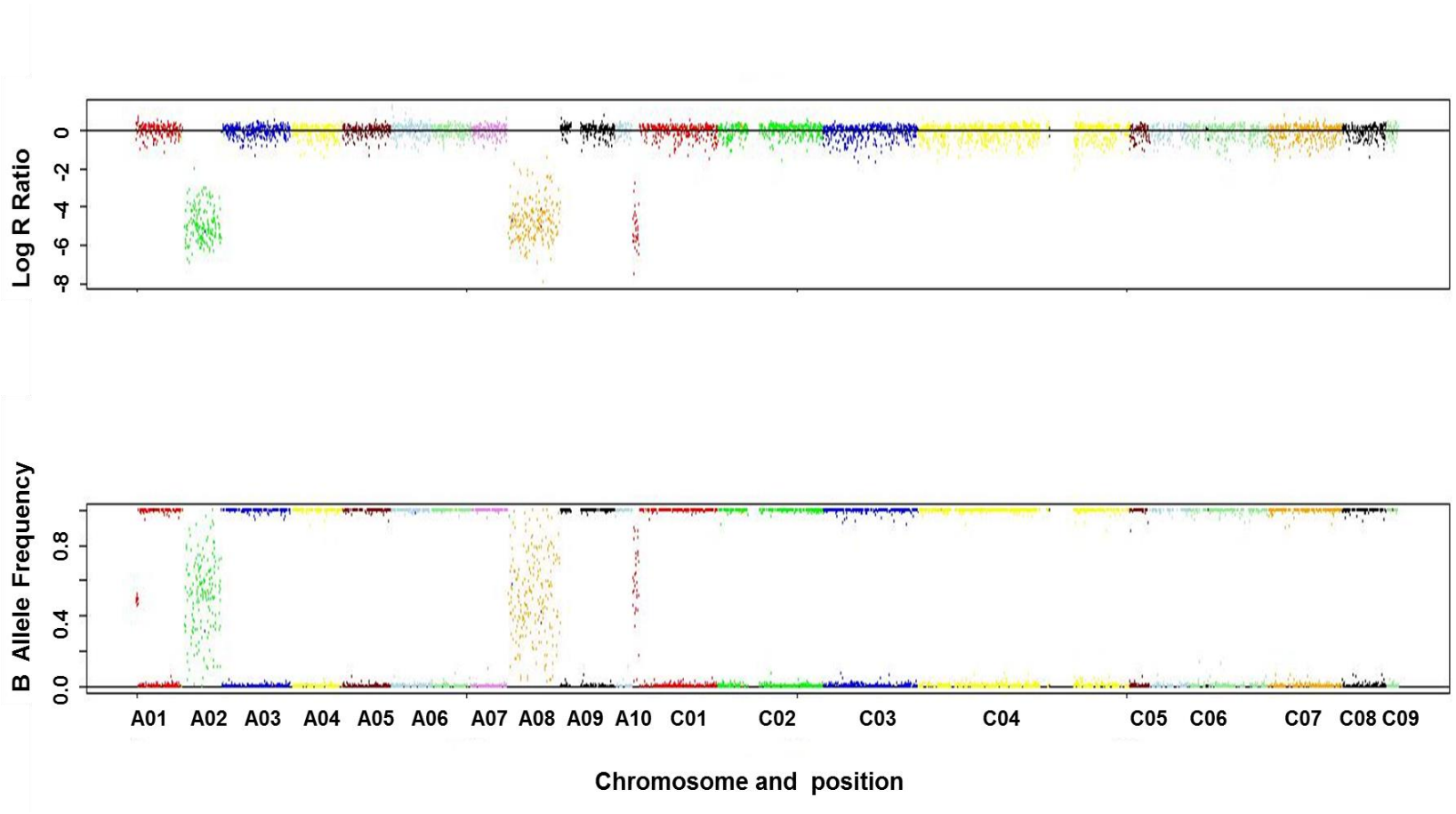


Figure 4.12 Copy Number Variation plots of MDL28_1 based on relative allele fluorescence ratios from Illumina Infinium *Brassica* 60K array SNP genotyping in MDL2 population shows a deletion in A02, A09 and C01 and a duplication in A01

4.4 Discussion

In this study we sought to determine genetic identity, fertility and meiotic behaviour in two generations of microspore-derived allohexaploid lines (MDL1 and MDL2). We used classical cytogenetics and *Brassic*a 60K Infinium Illumina array SNP genotyping data to test hypotheses relating to genetic integrity of microspore derived lines in meiotically unstable interspecific hybrids. Germination of the MLD2 progeny was generally poor, and several replanting trials had to be done (41.6% of the progeny sets planted germinated, while germination of lines within progenies varied from 20 - 67%). Fertility and stability characterisation of a microspore-derived allohexaploid *Brassica* population and subsequent SNP genotyping enabled us to effectively determine that non-homologous translocations may only rarely destabilise meiosis, but that meiotic instability commonly results in genetic variation in microspore-derived lines designed in efforts towards creating new allohexaploid *Brassica* species for agricultural benefit.

Firstly, we tested the hypothesis that all lines within potentially unstable allohexaploid *Brassica* microspore derived lines were identical. Based on *Brassica* 60K SNP genotype data, we found that all lines within two progeny sets were identical to each other and to their parents. However, this was not the case in the four remaining progeny sets, contrary to the general expectation that microspore derived lines are genetically stable and homozygous (Zhang et al. 2012). Although minimal allelic variation between parent MDL1 and the progeny MDL2 was observed, SNP genotyping analysis revealed the occurrence of fixed chromosome rearrangements (duplication/deletion events) also termed copy number variations (CNVs) as well as new duplication and deletion events contributing to genetic variation in the MDL2 population. The presence of univalents in MDL28 and MDL60 validates our secondary hypothesis that non-identical lines in this study are meiotically unstable. Further, we established the presence of deletion – duplication events which appears to be stably inherited from the parent to the progeny populations, including among the non-identical and meiotically unstable progeny sets.

Interestingly, although in most cases the presence of two alleles at each locus was indicated by the SNP copy number analysis, as expected, the presence of an extra allele was also observed in progeny sets MDL60 and MDL64, via observation of three alleles in a 2:1 ratio. This AAB or BBA clustering pattern is caused by the homeology between A and C genomes, which allows

SNP probes to hybridize to two different genomic positions in *B. napus* (Mason et al. 2017). Additionally, the function of CNVs in the resistance of plants to pests and pathogens is now being examined (Dolabatian et al. 2017). Despite this, the presence of genetic variation within MDLs is almost always undesirable, as disruption of homozygosity defeats the point of producing the MDLs in the first place. Therefore, careful consideration should be taken when producing MDLs from potentially unstable hybrid lines.

In this study, many chromosome deletions and duplications appeared to be stably inherited from the parent to the progeny populations, including the non-identical and unstable progeny sets. Some levels of multivalent formation occurring due to fixed chromosome rearrangements (whereby four chromosomes now share close homology) were also observed, while almost all fixed translocation regions were stably inherited from the MDL1 to MDL2. This could be due to selection for viability and fertility: we could be losing all the lines which had unstable inheritance of translocations due to our stringent fertility selection (58.4 % of the progeny sets and up to 33 – 80% of lines within progeny sets did not germinate).

Segregation of non-homologous translocation segments was evident in the MDL60 progeny, and surprisingly appeared to influence fertility. The line MDL60_7 had a unique deletion/duplication event involving chromosome A03/C03 in the MDL2 population, and had the highest seed set of 80: this line may be of interest for further evaluation of fertility and genomic stability. Overall, lower fertility was observed in MDL2 progeny compared to their MDL1 parents based on seed set data. This could support the theory that homeologous recombination occurring due to polyploidy destabilized the genomes, leading to additional future translocations and thus increasing instability (Gaeta and Pires 2010). Studies on allohexaploids from *B. carinata* × *B rapa* crosses found mixed results for intergenerational fertility: Howard et al. (1942) observed fertility to increase over several generations, while Iwasa et al. (1964) found fertility to be decreasing across generations. Mwathi et al. (2017) also observed decreased fertility and chromosome loss in *B. carinata* × *B. rapa* crosses in the second generation, with variable fertility in heterozygous second generation allohexaploids from the cross (*B. napus* × *B. carinata*) × *B. juncea*. A study on allohexaploids generated from different combinations found that *B. carinata* × *B. rapa* and *B. juncea* × *B. oleracea* crosses had higher fertility compared to allohexaploids arising either from natural and synthetic *B. napus* and *B. nigra* or from the sequential cross between *B. rapa, B. oleracea* and *B. nigra* (Zhou et al. 2016). From the results, generational fertility and genomic stability in

allohexaploid *Brassica* are very likely to be genotype-specific, which would also explain the significant variation in fertility and meiotic stability observed between MDL2 progeny sets in the present study.

In the MDL2 population, meiosis in the two progeny sets analysed was characterised by presence of univalents, with the greatest variation seen in the progeny set MDL60 (up to 5 univalents observed). Univalent formation at meiosis has been seen to result in low fertility and seed set in resynthesized lines (Qian et al. 2005). Genomic variants resulting from genomic rearrangements could also destabilize the karyotype, leading to aberrant meiotic behaviour and reduced fertility (Gaeta and Pires 2010; Szadkowski et al. 2011). In *Brassica* allohexaploid lines, the presence of irregular meiosis is thought to result almost entirely from an illegitimate pairing between homoeologs, as well as univalent inheritance and interactions between other non-homologous chromosome regions (Mason et al. 2015).

In the current study, chromosome loss (putatively mostly in the form of laggard univalent chromosomes) appeared prevalent; the number of chromosomes present was lower than expected in most lines. Given the chromosome complement of the ultimate parent plant (2n = 49; at least one copy of each homologous chromosome pair; Mason et al. (2015), MDLs should have contained between 49 and 54 chromosomes. However, the number of chromosomes observed was 40 – 48 (mode 48). Similar chromosome loss was observed by Zhou et al. (2016), who found extreme cases of C genome loss and a preferred retention of the B and A genomes. The loss of univalent chromosomes in MDLs in the present study comprises another source of genetic variation that can act to differentiate MDLs in the genomically unstable material.

Chapter 5: Production and meiotic assessment of *Brassica* hybrids from crosses between *B. juncea* and C genome species

5.1 Introduction

The genus *Brassica* contains six major oil and vegetable species of great economic and nutritional significance. The three-tetraploid species (*B. napus*, AACC*; B. juncea*, AABB*; B. carinata*, BBCC) are derived from pairwise genome combinations of the three-diploid species (*B. rapa*, AA; *B. oleracea*, CC; and *B. nigra*, BB*)* (Morinaga 1934; U 1935). The intergenomic relationship that exists between the *Brassica* species provides an opportunity to benefit from natural evolutionary processes to improve this species and to produce a new crop in this genus (Chen et al. 2011).

Wild species have attracted interest among breeders and geneticists seeking to improve crops species, particularly for the potential to use these species to introduce resistance to biotic and abiotic stresses (Lazáro & Aguinagalde 1998). *Brassica spinescens* and *Brassica maurorum* are reported to have resistance towards white rust, while *Brassica tournefortii* is resistant to aphids (Yao et al. 2012). Additionally, wild species are important in introducing genetic diversity where elite cultivars have undergone excessive inbreeding, are a source for cytoplasmic male sterility, hybrid seed production systems, and production of secondary metabolites (Mei et al. 2010). They are also a potential source of oil and condiments and tolerance to salt, cold and drought conditions (Rakow 2004; Branca and Cartea 2011).

Various studies have been conducted to determine crossability and relationship of wild species to crop species (Kianian and Quiros 1992; Lannér et al. 1997). Cytogenetic studies to examine the genomic relationship between *Brassica* crops and wild relatives (Kianian and Quiros 1992; von Bothmer et al. 1995; Rakow 2004) found that the wild forms of C genome species were significantly more inter-fertile with the cultivated forms, indicating a close relationship, while also constituting a cytodeme (Snogerup et al. 1990; Lannér et al. 1997). Snogerup et al. (1990) describes the morphology and provides a key to ease in the identification of the wild *Brassica* species: *B . macrocapa* is characterised by thin leaves, long petiolate and yellow flowers while *B. villosa* is characterised by leaves without wings at the base of the petiole, a sparsely divided lamina and long petiole. On the other hand, *B. cretica* is characterised (at subspecies level) by flowers ranging from white, light yellow to yellow, lower leaves can have long or short

petiolate, while *B. montana* is characterised by thin leaves, with flowers ranging from light to whitish yellow and often with a slight fragrance. Finally, *B. incana* is characterised by short petioles, with leaves often pinnatifid (arranged on each side of a common petiole).

A major challenge to accessing novel alleles in wild species is barriers to hybridisation that often lead to abortion of hybrid embryos, however, use of tissue culture and particularly ovary and ovule culture can often be used to rescue embryos successfully (Zhang et al. 2003; Bhat and Sarla 2004; Li et al. 2015). In Garg et al. (2007) hybrids between wild crucifers *Diplotaxis erucoides, B. mauromum* and *B. rapa* were developed using sequential ovary-ovule culture. However, a reciprocal cross was not possible, which may be because of incompatibility barriers.

Brassica juncea (AABB) is an important vegetable and oilseed crop in India and China, due to its relatively greater drought and heat tolerance than *B. napus* (Kaur et al. 2014; Gupta et al. 2015). *Brassica juncea* has many potential advantages over *B. napus* including: enhanced seedling vigour, blackleg resistance, shatter resistance, higher tolerance to drought and high temperature stresses (Oram et al. 1999). Also, *Brassica juncea* (cultivar "Xinyou 4") from China has also shown resistance to *Sclerotinia sclerotiorum* and *Leptosphaeria maculans* (Li et al. 2008). *Brassica oleracea* (CC) is a valuable vegetable crop for nutrition and health which contains multiple cultivar groups such as kales, cabbages, broccoli, cauliflower, brussels sprouts and kohlrabi. The *B. oleracea* "TO1000" cultivar in particular is useful for genetic studies due to its genetic homozygosity, rapid cycling and self-compatibility traits (Parkin et al. 2014).

Successful interspecific crosses between *B. juncea* and the other cultivated *Brassica* are more common and have been widely reported. However, *B. juncea* crosses with *B. oleracea* are less common (Stewart 2004; FitzJohn et al. 2007). Busso et al. (1987) report making trigenomic crosses for meiotic control and found the C. AB and AB.C combinations expressed mixed chromosome numbers including univalents and bivalents at meiosis. In a different study, Chen et al. (2006) reports making chimeras from *B. juncea* and *B. oleracea* for cytological analysis using *in vitro* graft-culture. Meanwhile, Li et al. (2015) produced trigenomic hybrids between *B. juncea* and *B. oleracea* using ovule culture followed by colchicine treatment for chromosome multiplication to produce allohexaploid germplasm. Zhou et al. (2016) on the other hand, produced *Brassica* allohexaploids between *B. juncea* and *B. oleracea* via embryo

rescue and treated with colchicine to double the chromosomes: euploids and aneuploids were produced and used to study chromosome loss. Weerakoon (2011) made interspecific hybrids using *B. juncea* and *B. oleracea* to produce trigenomic hybrids (ABC) without the use of ovule culture: however, crosses in the reciprocal direction with *B. oleracea* as the maternal parent and *B. juncea* as the paternal parent were unsuccessful in producing seeds.

In this study, I aim to use interspecific hybridisation to create hybrids between allotetraploid *B. juncea* (AABB), and wild C genome (CC) species *B. montana, B. cretica, B. macrocapa, B. villosa* and *B. incana* (2n = 18) (Lannér et al. 1997). Obtained hybrids would subsequently be cultured on ovule rescue media to overcome hybridization barriers. The triploid hybrids developed will be used to create an allohexaploid (AABBCC) *Brassica* with a goal to eventually build a genetically diverse foundation for a new species. This new species would potentially be adaptable over a wider geographical range and have a higher yield than its diploid and tetraploid progenitor crop species, while potentially overcoming genetic, meiotic instability and low fertility bottlenecks experienced in past research studies.

5.2 Materials and methods

5.2.1 Experimental material

Wild C cytodeme species *B. montana, B. macrocapa, B. villosa* and *B. incana* and two *B. juncea* genotypes (B578 and Xingyou 4) were obtained from the Australia Grains Genebank (AGG) in Horsham, Victoria (Table 5.1). *Brassica oleracea* (TO1000) seeds were obtained from Annaliese Mason (Justus Liebig University, Giessen, Germany). Five plants from each of the species *B. incana, B. montana, B. villosa, B. cretica, B. macrocapa, B. juncea* and *B. oleracea* (TO 1000) were sown at the University of Western Australia (UWA) Plant Growth Facilities (PGF) at 22 °C/20 °C day/night, from November 2015 - November 2016 for *B. juncea* and *B. oleracea* and until August 2017 for the wild species (Figure 5.1). Vernalisation was carried out at 4 °C to induce flowering.

Table 5.1 Germplasm used in interspecific hybridisation from the Australian Grains Genebank

Collection No.	Name	Taxon
AGG94704BRAS2	*Brassica montana* pourr.	*Brassica montana*
AGG95487BRAS2	UPM 6813	*Brassica montana*
AGG95516BRAS2	UPM 6563	*Brassica incana*
AGG95519BRS2	UPM 6595	*Brassica villosa*
AGG95524BRAS1	UPM 3819	*Brassica macrocapa*
ATC 95640	Xinyou 4	*Brassica juncea*
ATC 90333	PI 257240/ B578	*Brassica juncea*
AGG (Unknown)	*Brassica cretica*	*Brassica cretica*

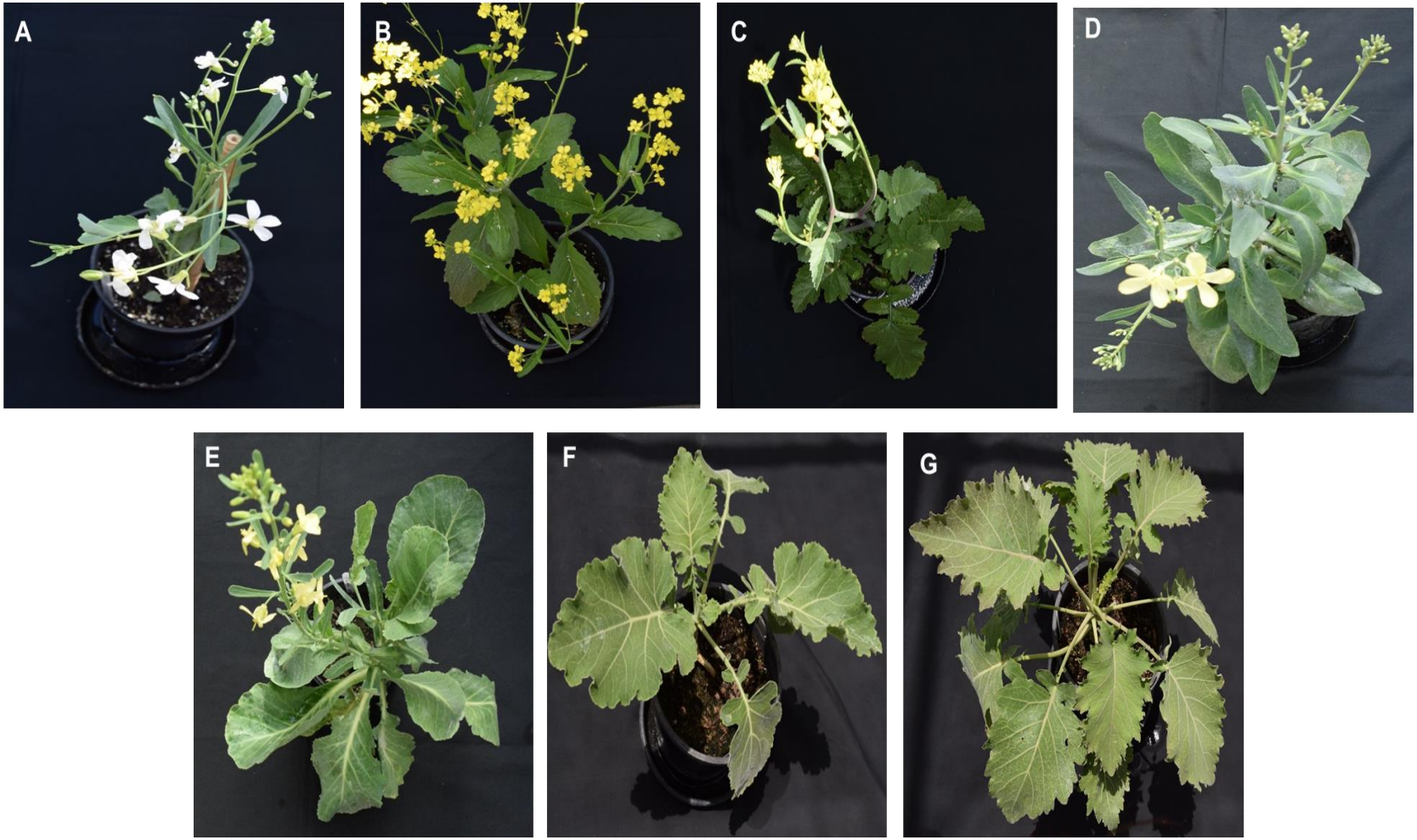


Figure 5.1 Images of germplasm used in interspecific hybridisation A: *B. oleracea* (TO1000) B: *B. juncea* (B578) C: *B. incana* D: *B. cretica* E: *B. montana* F: *B. villosa* G: *B. macrocapa*

5.2.2 Embryo culture for wide hybridisation

Interspecific hybridisation between each pair of lines using each as both the male and female parent (reciprocal) was carried out using hand pollination at flowering. Fertilized ovules were harvested at 14 and 21 days after pollination. Ovules were surface sterilised by soaking in 70% ethanol for 1 minute, followed by a 15-minute incubation in 10% sodium hypochlorite and subsequently three rinses with sterile water for 5 minutes each time (prepared as described in sections 2.3, 2.4 and 2.5). The ovules were cultured on ovule rescue media and allowed to grow for 10 - 15 days at 25 °C under a 16/8 hrs day/night regime (prepared as described in section 2.15). After two weeks, the ovaries were dissected and cultured on regeneration media (prepared as described in section 2.16), (Figure 5.2). Ovaries were left in complete darkness until germination. Plants obtained were multiplied by sub-culturing in a series of multiplication media (prepared as described in section 2.17).

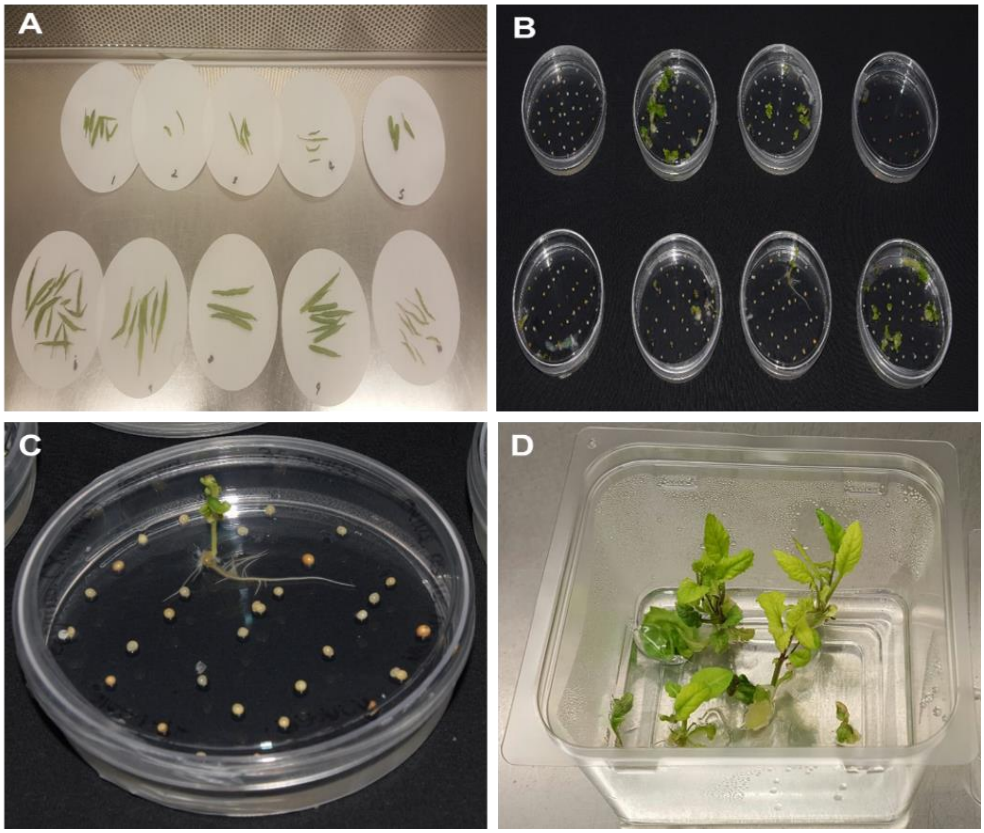


Figure 5.2 A: wild C genome species/*B. oleracea* × *B. juncea* ABC hybrid siliques; B and C: ovules in ovule rescue media and D: plantlets in regeneration media

5.2.3 Chromosome multiplication of putative ABC hybrid plants

Putative ABC hybrid plants obtained from crosses between wild C genome species/*B. oleracea* and *B. juncea* were regenerated (*in-vitro)* in culture using colchicine media (prepared as described in section 2.18) (Figure 5.3). A set of controls untreated by sub-culturing plantlets were regenerated on the same media without colchicine. Putative hybrids obtained were cultured on rooting media (prepared as described in section 2.19) before being transferred to potting soils for hardening in a humid environment for one week. Plants obtained were transferred to glasshouses at The University of Western Australia for phenotypic, fertility, ploidy and meiotic analysis.

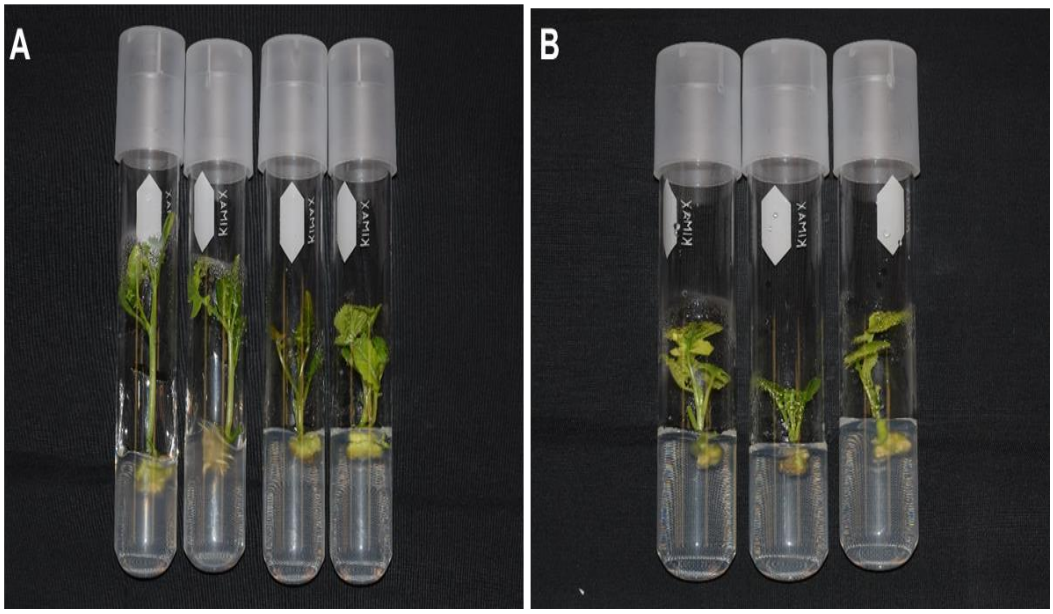


Figure 5.3 A, B: plantlets resulting from interspecific crosses between wild C genome species/ *B. oleracea* × *B. juncea* crosses in colchicine treatment media

5.2.4 Pollen fertility and seed set

Ripe floral buds (almost open) were collected during early morning hours. Pollen grains were assessed by squashing anthers in 1% acetocarmine (prepared as described in section 2.2) on glass microscope slides. The observation was carried out by using a compound light microscope. Pollen grains that were round and darkly stained were assumed to be viable, whereas unstained and shrivelled pollen were assumed to be nonviable. On average 300 pollen

grains were scored from at least two flowers obtained for each line. The racemes of each plant were enclosed in bread bags to encourage self-pollination.

5.2.5 Meiotic chromosome observations

Floral buds were collected and fixed in Carnoy's II solution (ethanol: chloroform: acetic acid 6:3:1) (prepared as described in section 2.1) for up to 48 hours and then stored in 70% ethanol (prepared as described in section 2.4) at 4 °C. Anthers were prepared by staining followed by squashing in a drop of 1% acetic acetocarmine (prepared as described in section 2.2) solution on glass microscope slides. The minimum number of PMCs counted per plant was ten cells, the maximum was 30 cells, while the average was 20 PMCs. Observations of the pollen mother cells (PMCs) were carried out at meiosis using a ZEISS light microscope (bright field phase contrast at 400- and 1000-times magnification) and images captured using the Axio Vision Imaging system (Release 4.8.1) at the University of Western Australia.

5.2.6 Flow cytometry analysis

Suspension of nuclei from the *B. oleracea* × *B. juncea* ABC hybrids, and for *B. incana*× *B. juncea* ABC hybrids was prepared for flow cytometry analysis (prepared as described in section 2.14). Nuclear DNA content was measured on a BD FACS Canto II (BD Biosciences) flow cytometer, using FACS Diva V6.1.1 operating software. Propidium iodide dye (prepared as described in section 2.6) was excited with a 488 - nm (blue) laser and PI emission collected with a 585/42 (564 - 606 nm) \bandpass filter. Experimental data were analysed using FlowJo software V7.2.5 (Tree Star Inc., Ashland, OR), to determine the mean size of the gate and sample peaks.

Flow cytometry was used to validate if true hybrids (2n = 3*x* = ABC) or colchicine-doubled true hybrids (2n = 6*x* = AABBCC) were produced from the diploid (2*x*) × tetraploid (4*x*) reciprocal cross combinations. Ploidy *x* value was determined relative to the parent controls, with an expected value of 2n = ABC = 1.0 calculated by the formula (*B. oleracea* mean + *B. juncea* mean)/2 for *B. oleracea* × *B. juncea* ABC hybrids, and (*B. incana* mean + *B. juncea* mean)/2 for *B. incana*× *B. juncea* ABC hybrid.

5.2.7 Chromosome multiplication of *B. oleracea* × B*. juncea* (ABC) triploid hybrids

Six hybrid plants from interspecific hybridisation of *B. oleracea* × *B. juncea* (JO 1, JO 2, JO 2.2, JO 3, JO 4, JO 5), were transferred to bigger pots to allow growth into bigger healthy plants. A new set of cuttings for each plant was propagated using rooting hormone gel (Yates, Bunnings) containing 3 g/L Indole 3-butyric acid. These new cuttings were replicated to produce nine healthy plants per hybrid line (54 in total). Six different colchicine treatments were carried out on six different branches for each plant (control 0, 0.05, 0.1, 0.15, 0.2 and 0.25% w/v in double distilled water (Pradhan et al. 2010). The treatments were covered in aluminium foil and tagged (a, b, c, d, e, f, g and h). An untreated plant (control) was retained for each hybrid. The colchicine application treatment was repeated on the same meristem twice a day for three successive days at 9 a.m. and 3 p.m.

5.3 Results

5.3.1 Overcoming hybridisation barriers

Interspecific hybridisation was carried out after hand emasculation between five plants each of *B. juncea* with *B. oleracea*, *B. incana*, *B. cretica* and *B. montana* in reciprocal crossing directions; thereafter, ovule rescue was carried out to overcome hybridisation barriers. *Brassica juncea*, *B. incana* and *B. oleracea* flowered after three months, while *B. cretica* and *B. montana* flowered after five months. The *B. macrocapa* and *B. villosa* C genome species did not flower despite vernalisation at 4 °C from November 2015 – February 2016 for a period of ten weeks. The optimum time to harvest pods was identified as 15 days after pollination (DAP) for *B. juncea* pollinations with *B. incana* and *B. oleracea* and 21 DAP in *B. juncea* pollinations with *B. montana* and *B. cretica* interspecific hybrids.

A total of 447 flowers were pollinated for *B. juncea* × *B. incana* crosses in both directions using two *B. juncea* genotypes (Xinyou 4 and B574). A total of 326 ovules were produced and cultured in media. Up to 60 plants that regenerated were sub-cultured through colchicine media and subsequently in multiplication media. Six plants survived while the rest succumbed to viral and bacterial contamination. The crossability for *B. juncea* × *B. incana* was 0.02 in both directions (calculated by dividing the number of plants in final multiplication media/ the total number of flowers pollinated) (Table 5.2). The surviving putative *B. juncea* × *B. incana* (AB.C) hybrids were labelled IJ 1, IJ 2, IJ 3, IJ 4.1, IJ 4.2, and IJ 5.1. The flowers in these plants were yellow in colour like those of their parents while the leaves and plant morphology were intermediate between the two parents (Figure 5.4).

A total of 85 flowers were pollinated for *B. oleracea* × *B. juncea* crosses. A total of 35 ovules were produced and cultured in media. Six plants regenerated and were sub-cultured in colchicine media and subsequently in multiplication media. The crossability for these species was 0.07 (calculated by diving the number of plants in final multiplication media/ the total number of flowers pollinated) (Table 5.2). Six putative *B. oleracea* × *B. juncea* hybrids (C. AB) survived and were labelled: JO 1, JO 2, JO 2.2, JO 3, JO 4 and JO 5. The flowers of these plants were white in colour, resembling the maternal parent *B. oleracea,* while the leaves were broader resembling the parental plant *B. juncea*; the leaf edges were smoother also resembling *B. oleracea* (Figure 5.4).

A total of 150 flowers were pollinated for interspecific crosses between *B. juncea* and *B. cretica* which produced up to ten surviving ovules; however, these did not produce any plants on regeneration. A total of 70 flowers were pollinated for interspecific crosses between *B. juncea* and *B. montana*, but the crosses were not successful in producing any surviving ovules.

Table 5.2 Crossability between *Brassica juncea* and *B. oleracea*/wild C genome species

Crosses	Flowers pollinated(a)	Ovules cultured	Plants regenerated	Hybrids in MS media (b)	Crossability (b/a)
B. juncea (Xinyou 4) ♀ × *B. incana* ♂	300	221	60	6	0.02
B. incana ♀ × *B. juncea* (Xinyou4) ♂	75	40	0	0	0
B. juncea (B578) ♀ × *B. incana* ♂	57	45	0	0	0
B. incana ♀ × *B. juncea* (B578) ♂	15	20	0	0	0
*B. juncea (*Xinyou 4*)* ♀ × *B. montana* ♂	55	0	0	0	0
B. montana ♀ × *B. juncea (*Xinyou 4*)* ♂	15	0	0	0	0
B. juncea (Xinyou 4) ♀ × *B. cretica* ♂	15	10	0	0	0
B. cretica ♀ × *B. juncea* (Xinyou 4) ♂	75	0	0	0	0
B. juncea (JN4-09) ♀ × *B. cretica* ♂	35	0	0	0	0
B. cretica ♀ *x B. juncea* (JN4-09) ♂	25	0	0	0	0
B. juncea (JN4-09) ♀ × *B. montana* ♂	35	0	0	0	0
B. juncea (B578) ♀ × *B. oleracea* (TO1000) ♂	45	0	0	0	0
B. oleracea (TO1000) ♀ × *B. juncea* (B578) ♂	85	35	6	6	0.07

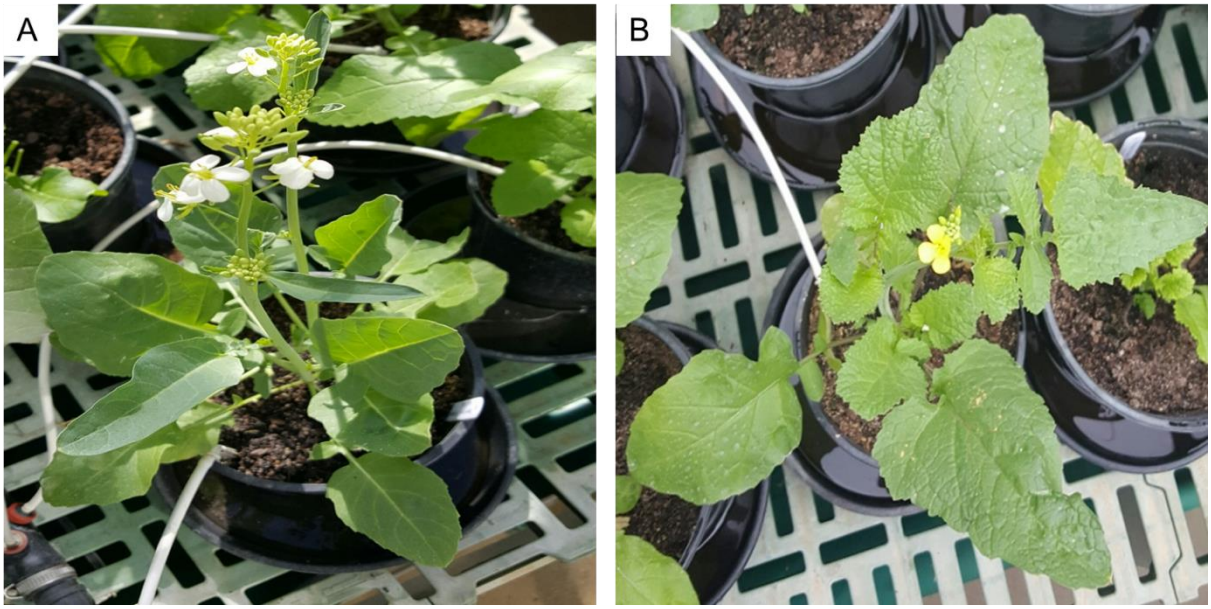


Figure 5.4 Images of the resulting putative hybrids plants from interspecific hybridisation: A: ABC hybrid JO 2.2 (*B. oleracea* × *B. juncea*) B: ABC hybrid IJ 5.1 (*B. juncea* × *B. incana*)

5.3.3 Confirmation of hybridity by ploidy level and fertility

Pollen fertility in the parent controls ranged from 88 – 95%, with the *B. juncea* and *B. incana* parents also setting hundreds of seeds (Table 5.3). Pollen fertility in the *B. oleracea* × *B. juncea* hybrids was generally low, with a range of 2 – 10% and an average fertility of 5.8% (Figure 5.5, Table 5.3). None of these *B. oleracea* × *B. juncea* hybrids formed pods or set any seeds, suggestive of sterility. By contrast, the *B. juncea* × *B. incana* hybrids produced 20 – 200 seeds each and showed 75 - 91% pollen viability (Table 5.3).

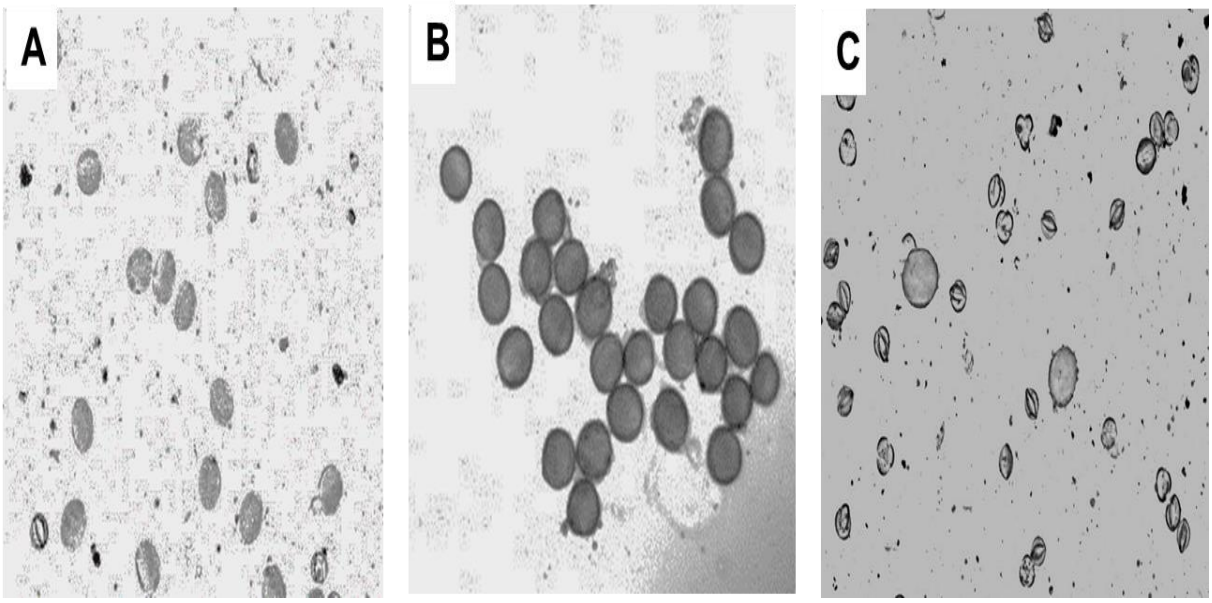


Figure 5.5 A: Pollen fertility in A: *B. oleracea* B: *B. juncea* C: *B.oleracea* × *B. juncea* ABC JO 2.2 hybrid (Magnification 100 × objective lens)

The measurement of nuclear genome content using flow cytometry shows that ploidy levels in the *B. oleracea* × *B. juncea* hybrids were intermediate between the ploidy levels of the *B. oleracea* and *B. juncea* parents (Figure 5.6, Figure 5.7) ranging from 0.86 – 0.98 of the expected ploidy values for 2n = ABC genome complements (Table 5.3). Unexpectedly, the *B. incana* parent showed a ploidy level nearly twice that of the *B. oleracea* parent, very similar to that of the *B. juncea* parent. All putative hybrids between *B. incana* and *B. juncea* also showed similar ploidy levels to those of their two parents (Figure 5.6, Figure 5.8).

Based on the fertility and ploidy data, all *B. oleracea* × *B. juncea* hybrids (JO 1, JO 2, JO 2.2, JO 3, JO 4 and JO 5) were confirmed to true hybrids, and to most likely have the genome composition 2n = ABC. Unfortunately, the *B. incana* parent genotype was predicted not to be *B. incana*, but most likely instead a *B. juncea* accession which was previously misidentified. Hence, all *B. juncea* × *B. incana* hybrids were predicted to be intraspecific hybrids between two *B. juncea* accessions, with genome complements of 2n = AABB.

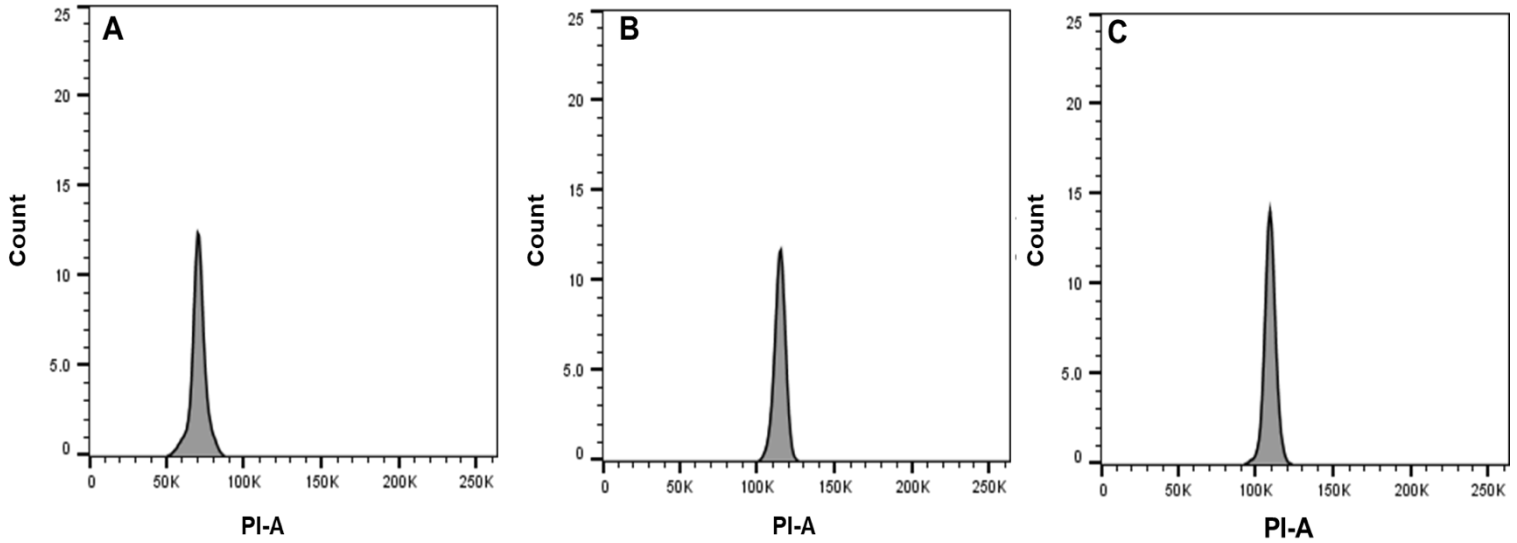


Figure 5.6 Flow cytometry ploidy graphs for parents A: *B. oleracea* (TO1000) B: *B. juncea (*B578*)* C: *B. incana*

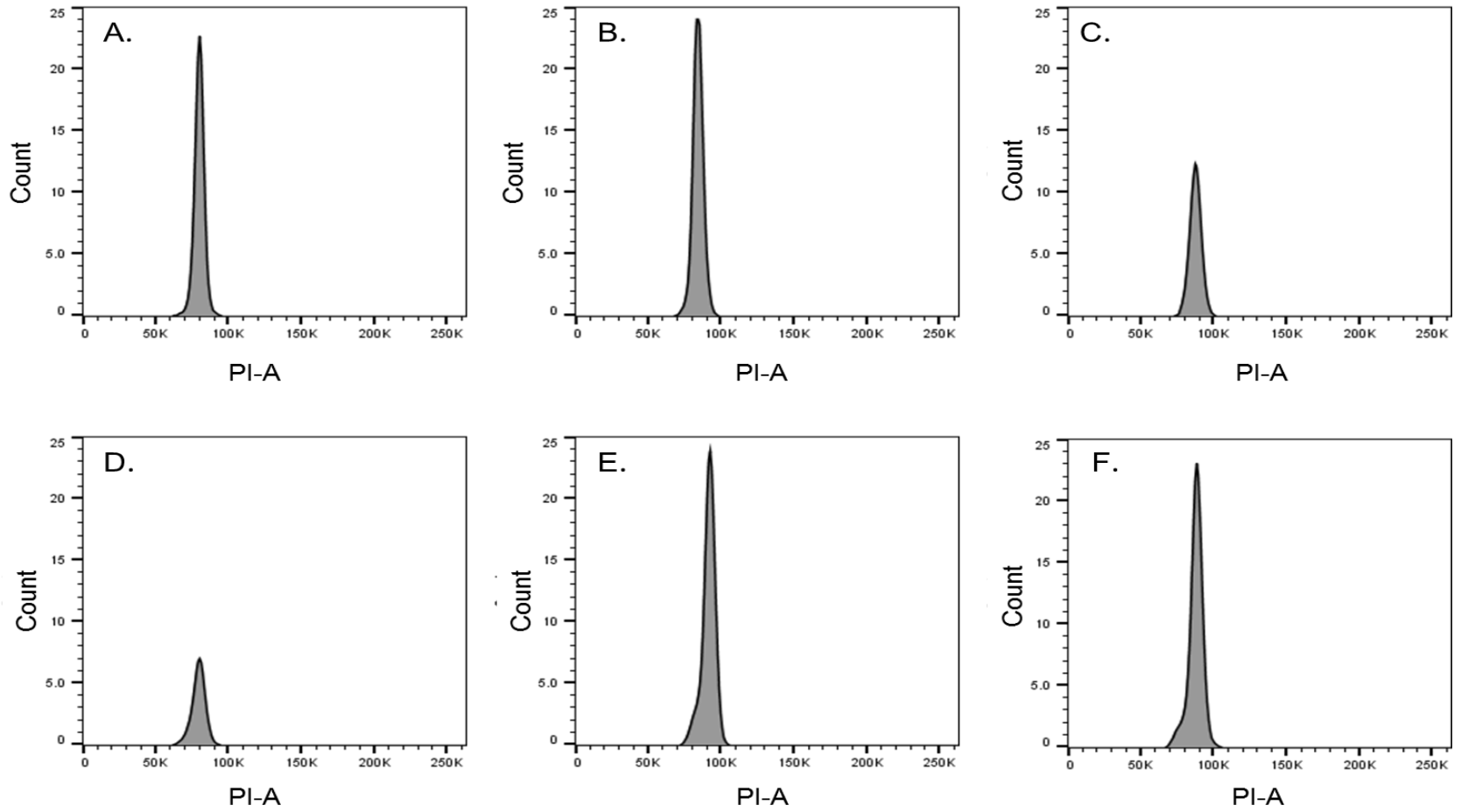


Figure 5.7 Flow cytometry ploidy graphs for putative *B. oleracea* × *B. juncea* ABC hybrids A: JO 1 B: JO 2 C: JO 2.2 D: JO 3 E: JO 4 F: JO 5

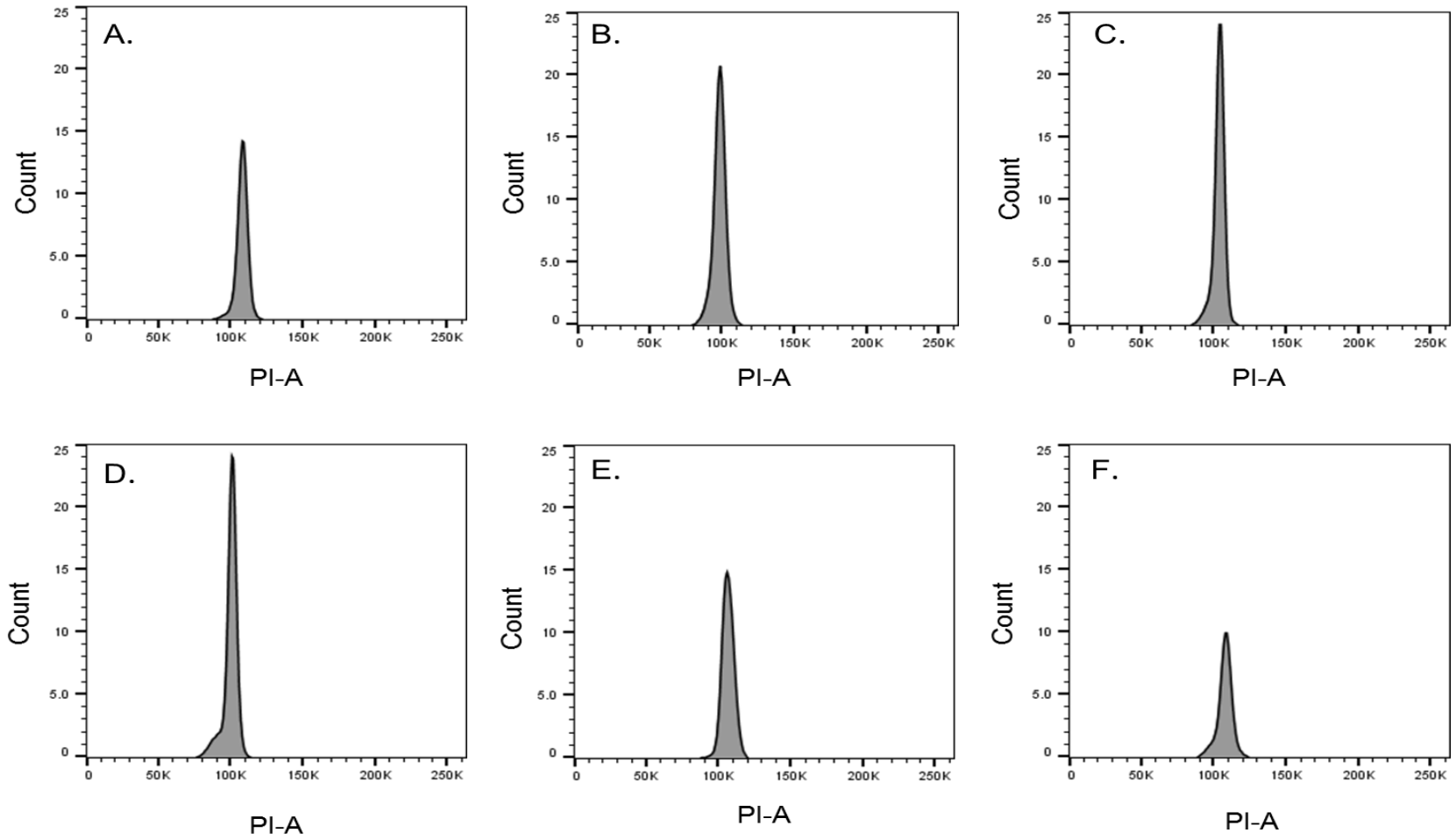


Figure 5.8 Flow cytometry ploidy graphs for putative *B. incana* × *B. juncea* ABC hybrids A: IJ 1 B: IJ 2 C: IJ 3 D: IJ 4.1 E: IJ 4.2 F: IJ 5.1

Table 5.3 Fertility and ploidy levels of putative interspecific *Brassica* triploids (2n = ABC) produced from the cross *B. oleracea* × *B. juncea* and *B. juncea* × *B. incana* and their parents

Plant name	Maternal parent	Maternal genotype	Paternal parent	Paternal genotype	% Pollen viability	Seed production	Flow DNA Mean
JO 1	*B. oleracea*	T01000	*B. juncea*	Xinyou4	2	0	79329
JO 2	*B. oleracea*	T01000	*B. juncea*	Xinyou4	6	0	83360
JO 2.2	*B. oleracea*	T01000	*B. juncea*	Xinyou4	5	0	86288
JO 3	*B. oleracea*	T01000	*B. juncea*	Xinyou4	4	0	86706
JO 4	*B. oleracea*	T01000	*B. juncea*	Xinyou4	10	0	79001
JO 5	*B. oleracea*	T01000	*B. juncea*	Xinyou4	8	0	90490
IJ 1	*B. juncea*	B578	*B. incana*	6563	90	20	107094
IJ 2	*B. juncea*	B578	*B. incana*	6563	85	120	97655
IJ 3	*B. juncea*	B578	*B. incana*	6563	75	90	102259
IJ 4.1	*B. juncea*	B578	*B. incana*	6563	85	150	99117
IJ 4.2	*B. juncea*	B578	*B. incana*	6563	91	180	105881
IJ 5.1	*B. juncea*	B578	*B. incana*	6563	75	200	106655
B. juncea					88	450	113846
B. oleracea					95	100	70089
B. incana					90	300	108369

5.3.4 Chromosome multiplication of ABC hybrids to produce *B. oleracea* × *B. juncea* allohexaploids

Confirmed *B. oleracea* × *B. juncea* (3*x*) hybrids were treated with colchicine of concentrations ranging from 0 - 2.5% (w/v). Changes were observed in pods, leaves and stems relative to the untreated control. There was pod discoloration from a healthy green colour to brown, a curling and tightening of leaves as well as deformity and thickening of stems (Figure 5.9).

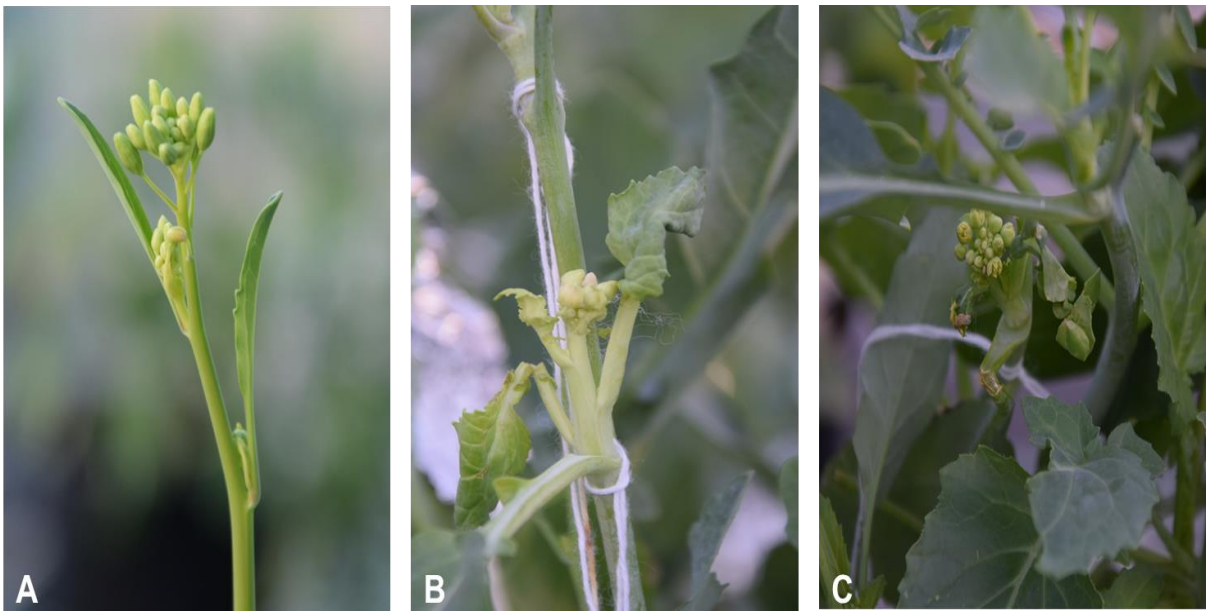


Figure 5.9 A: normal pods, leaves and stem before colchicine treatment B, C: deformed pods, thickened stems and curled leaves after colchicine treatment in *B. oleracea* × *B. juncea* hybrids

Plants treated with different concentrations of colchicine all showed some degree of pod setting and seed setting. A total of 17 pods containing 16 seeds were harvested from plants in the 0.05% colchicine treatment condition. Total of 36 pods containing 35 seeds were harvested from plants in the 0.1% colchicine treatment condition. Total of 42 pods containing 62 seeds were harvested from the 0.15% colchicine treatment condition. Total of 28 pods containing 58 seeds were harvested from the 0.2% colchicine treatment condition, and 36 pods containing 29 seeds were harvested from the 0.25% colchicine treatment condition. In total, 200 seeds were harvested (Figures 5.10, 5.11). Up to 140 seeds from the S_0 generation were planted, of which 94 S_1 generation plants survived to flowering.

Phenotypic analysis of the putative allohexaploid S_1 generation showed an increased thickness of the main stem, an increase in leave size (span) and a difference in leaf surface texture and margins compared to ABC hybrids and parents. A more compact bud setting was observed in the *B. oleracea* × *B. juncea* allohexaploid compared to ABC hybrids (Figures 5.12, 5.13). Pollen fertility ranged from 7 - 84% with an average of 57% within the S_1 allohexaploid population. Fertility and genome stability characterization of the allohexaploid S_1 generation is planned and will be characterised in a future study.

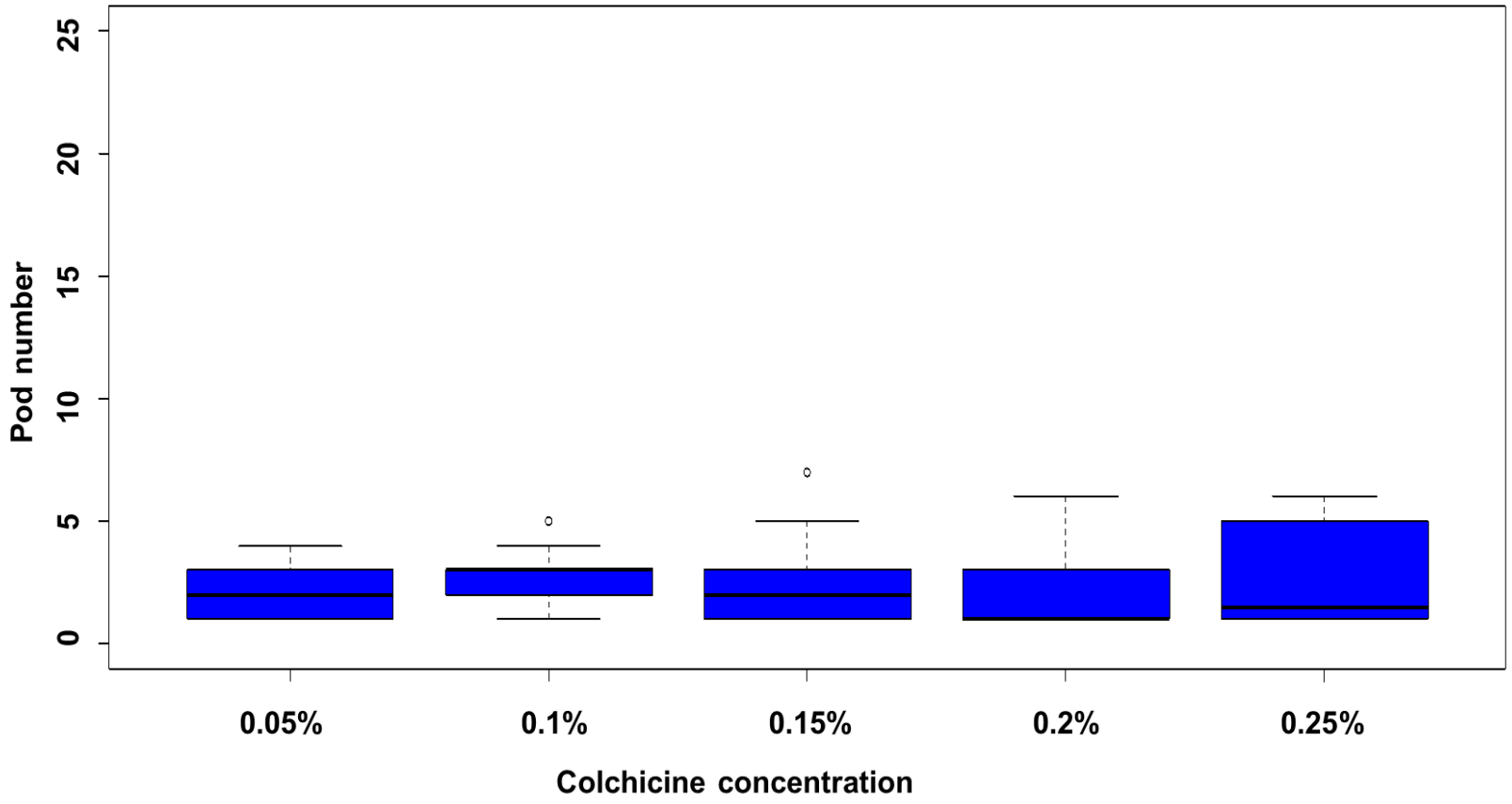


Figure 5.10 Pod setting of *Brassica* triploid hybrids (2n = ABC) from the cross *B. oleracea* × *B. juncea* after treatment with different concentrations of colchicine to putatively double ploidy level.

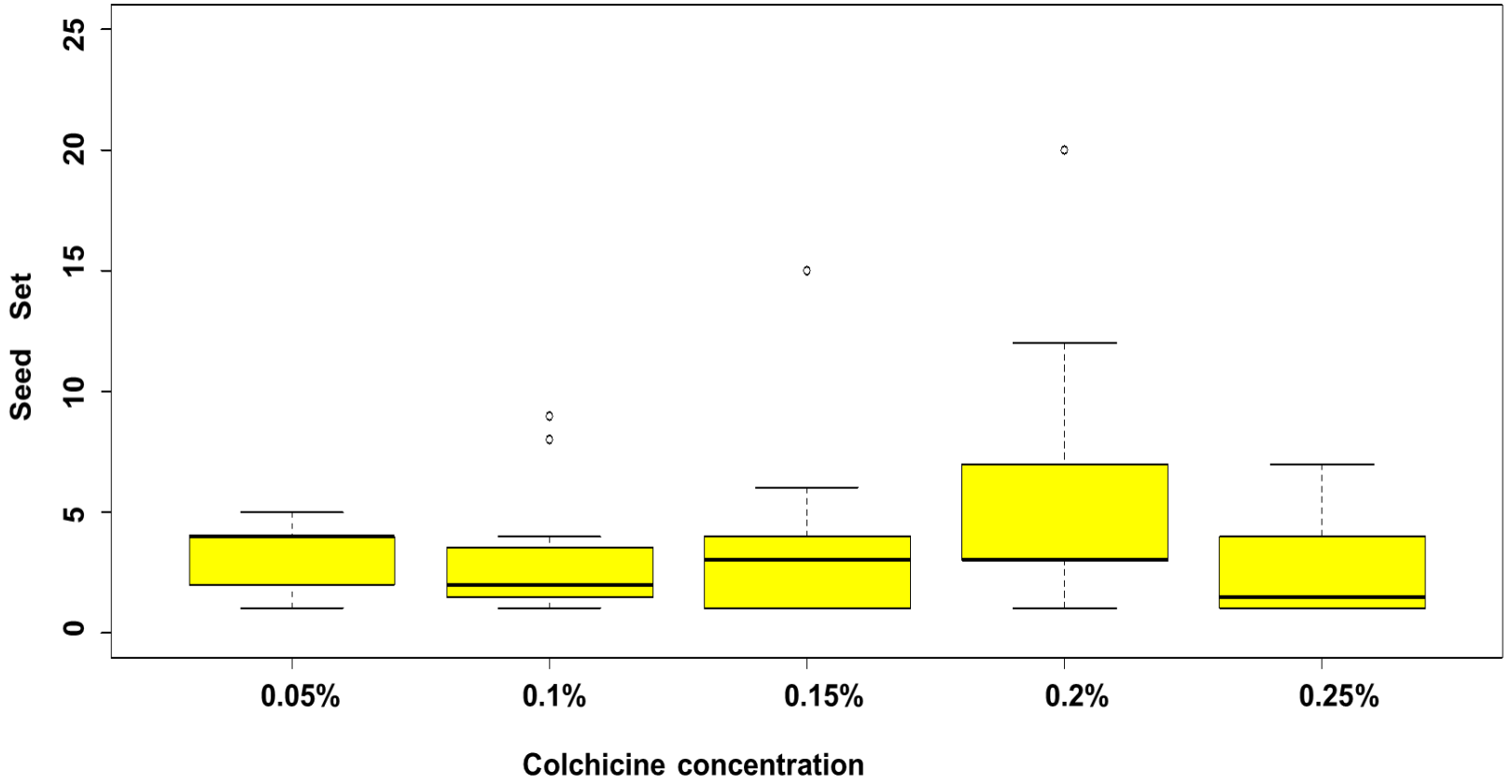


Figure 5.11 Seed setting of *Brassica* triploid hybrids (2n = ABC) from the cross *B. oleracea* × *B. juncea* after treatment with different concentrations of colchicine to putatively double ploidy level.

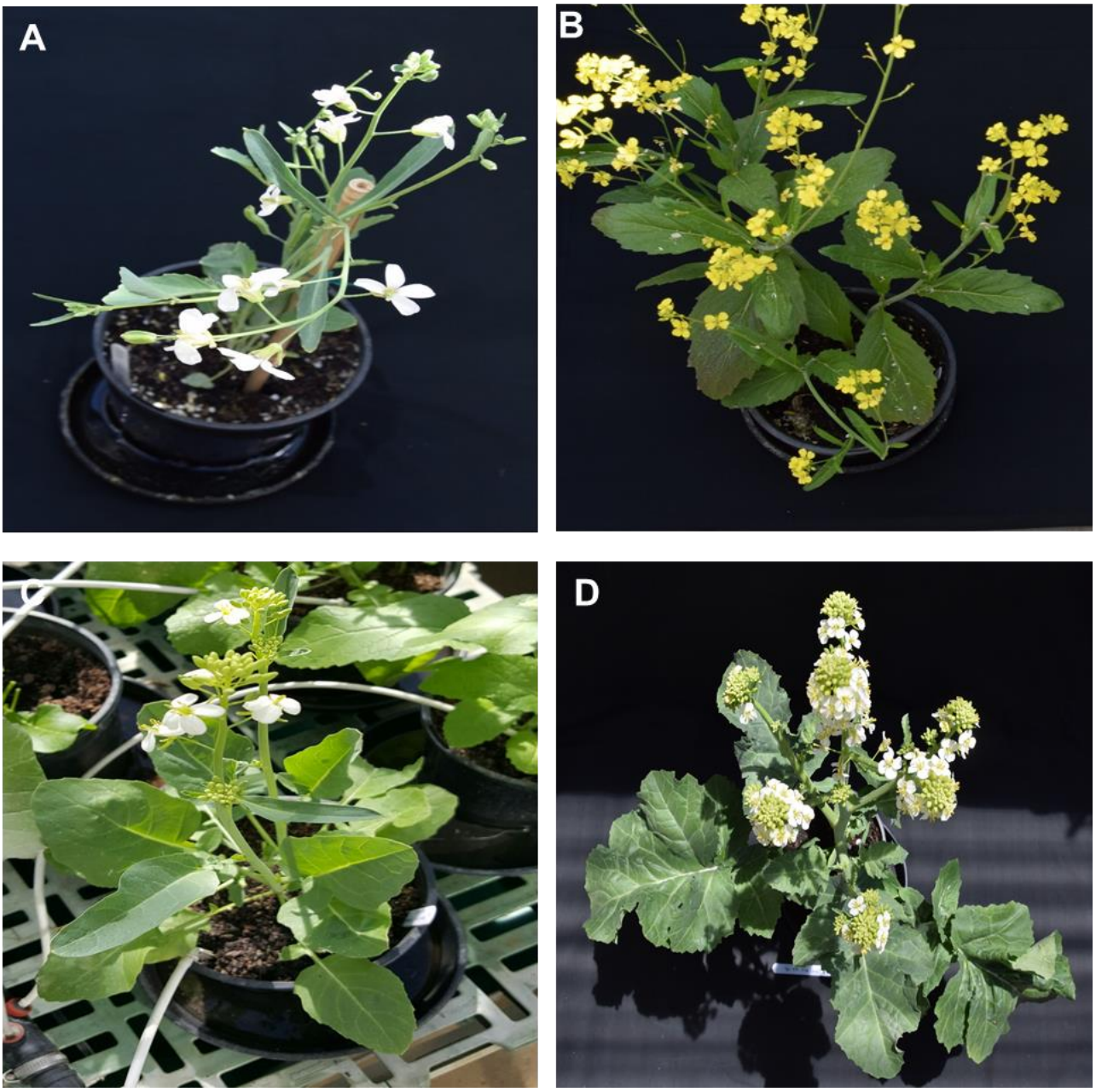


Figure 5.12 Images of *B. oleracea* parent B*: B. juncea* parent C: *B. oleracea* × *B. juncea* ABC hybrid and D: *B. oleracea* × *B. juncea* allohexaploid

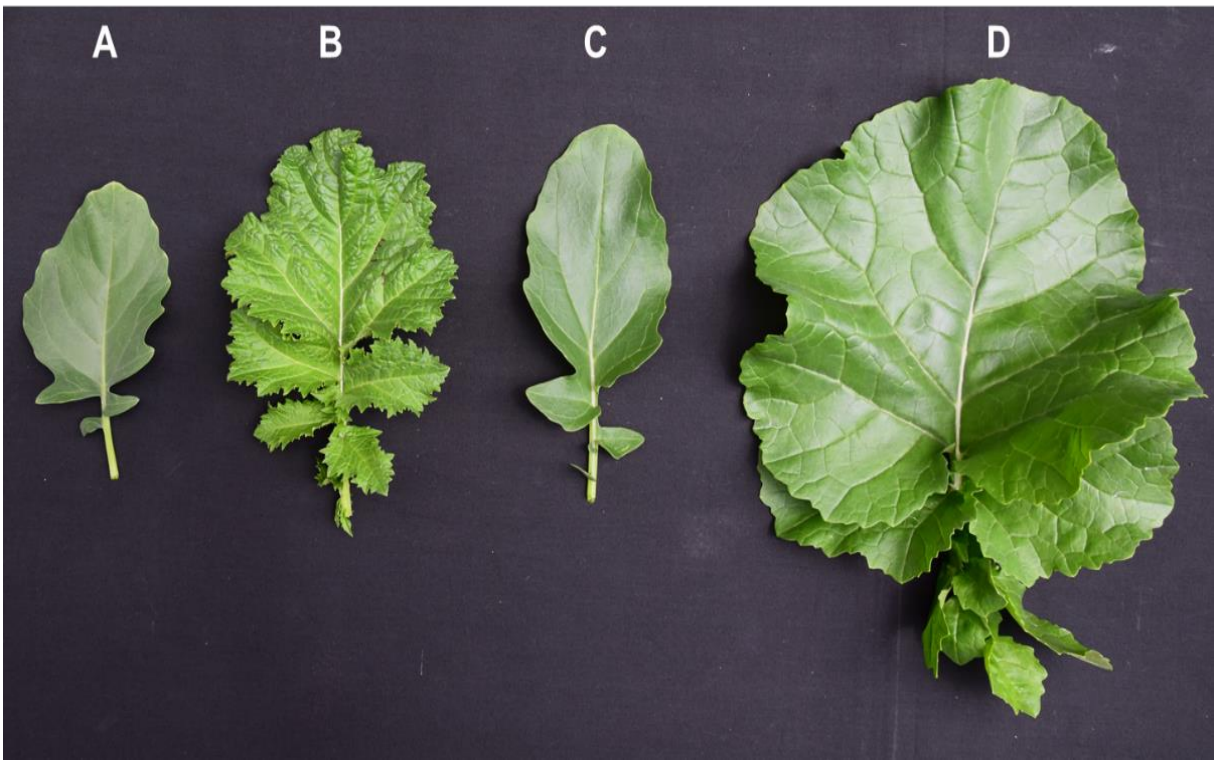


Figure 5.13 Leaves of A: *B. oleracea* "TO1000" B: *B. juncea* "B578", C: the ABC triploid hybrid produced from the cross *B. oleracea* × *B. juncea* and D: *B. oleracea* × *B. juncea* allohexaploid produced by chromosome doubling of the triploid hybrid

5.4 Discussion

The aim of this chapter was to increase genetic diversity in a new allohexaploid species through synthesis of novel ABC trigenomic hybrids from the wild C genome species/*B. oleracea* and *B. juncea*, ovule rescue to overcome interspecific hybridisation barriers. Hand pollination between two genotypes of *B. juncea* (Xingyou 4 and B578*)* with *B. oleracea* (TO1000) and wild C genome species *B. incana*, *B. montana* and *B. cretica* were performed (747 total bud pollinations, average 62.25 per cross combination) in both cross directions. However, only two cross combinations produced viable plants. The *B. juncea* by *B. incana* crosses produced six putative hybrids. Unexpectedly, ploidy testing revealed that the *B. incana* parent had very similar ploidy levels to *B. juncea* (tetraploid). Further, while these hybrids resembled both parents from phenotypic observations, very high pollen fertility and seed set were observed, similar to parent levels. It is not uncommon for accessions in the *Brassica* genus to be wrongly classified by species in germplasm collections (Mason et al. 2015), due to the many phenotypic similarities between species. Hence, we predict that the *B. incana* accession sourced from the Australian Grains Genebank for this study was in fact *B. juncea*: resulting progeny were predicted to be intraspecific *B. juncea* hybrids.

The second successful cross combination with *B. oleracea* and *B. juncea* was successful with a crossability ratio of 0.07 (number of plants in final multiplication media/ the total number of flowers pollinated). Up to seven triploid hybrids (2n = ABC) from 85 flowers and 35 cultured ovules were produced. Schelfhout et al. (2006) reports that interspecific hybrids have more success when the higher ploidy parent is the female parent and the lower ploidy parent is the male parent, in this case however, success was only observed in the reverse direction with *B. oleracea* (CC) as the maternal parent and *B. juncea* (AABB) as the paternal parent. Flow cytometry analysis for these *B. oleracea* × *B. juncea* hybrids revealed ploidy levels to be reasonably close to what we would expect for an ABC hybrid from a *B. oleracea* × *B. juncea* cross. We observed that the DNA content from the ploidy analysis was higher than what we would expect for *B. oleracea* (2*x*) but lower than *B. juncea* (4*x*), suggestive of 3*x* triploids. Additionally, phenotypic observations, the low pollen fertility and lack of seed setting confirmed these to be ABC hybrid plants.

Interspecific crosses between *B. juncea* and the wild species *B. montana* and *B. cretica* were unsuccessful, possibly because of earlier–stage incompatibilities involved in the ovule

fertilization stage. Also, this may be occurring as a result of genotype-specific infertility, which is caused by pre- or post-zygotic barriers at the stigma, style, or ovary among interspecific hybrids (Schelfhout et al. 2006). The *B. maurorum* and *B. villosa* accessions did not flower despite vernalization of up to 10 weeks: these particular wild C genome species may require vernalization for longer periods to enable flowering to take place. Additionally, the number of crosses that could be carried out with these species was hindered by poor seed germination of the starting material and low numbers of buds produced at flowering. Only five plants germinated and were used for crossing for each of the *B. montana, B. cretica, B. incana, B. oleracea* and *B. juncea* species, with one genotype per species. Successful production of a larger number of flowering plants from each of the wild species, as well as incorporation of diverse wild accessions, could contribute to overcoming genotypic incompatibilities and increase the chances for successful hybridisation.

Although reports of successful interspecific hybridisation between *B. juncea* and wild species are not common, *B. juncea* has been described as an ideal species to use in different crossing experiments because of its ability to produce reasonable seed set with other *Brassica* species in both cross directions (Stewart 2004; FitzJohn et al. 2007). However, the success of previous hybridisation attempts using *B. juncea* has been variable. Synthesis of trigenomic hybrids between the cultivated *Brassica* allotetraploids and *Brassica fruticulosa* was unsuccessful with *B. juncea* and *B. napus* crosses, although hybrids were produced using *B. carinata* (Chen et al. 2011). Meanwhile, Yao et al. (2010) produced sterile interspecific hybrids between wild species *B. maurorum* and three cultivated *Brassica* allotetraploids *B. napus*, *B. juncea* and *B. carinata*; subsequent chromosome doubling led to varying fertility with *B. juncea* reported to have a good seed set.

Wild taxa have been of interest to plant breeders and geneticists as a potential resource for breeding experiments, and 2n = 18 *Brassica* species and their wild relatives can be easily crossed (Snogerup et al. 1990). Studies conducted on the *B. oleracea* cytodeme also show great genetic diversity (Mei et al. 2010), supporting the use of these species as progenitor germplasm for crop improvement and in the production of a new, diverse allohexaploid crop species. However, a majority of the wild *Brassica* species have proven difficult to use in research programs, mainly due to their extended vegetative phase (Branca and Cartea 2011). In this study, we also found difficulties in inducing flowering in the wild C genome species. Also, interspecific crosses often have strong hybridisation barriers and require time-consuming

selection following rapid segregation, while offspring in early generations have poor fertility and genome stability (Zhou et al. 2016). These hybridisation barriers can however be overcome by using embryo rescue to create viable hybrids. Li et al. (2015) report production of a trigenomic hybrid using *B juncea* and *B. oleracea* followed by colchicine treatment to produce an allohexaploid, with subsequent characterization using molecular markers and flow cytometry. In a study by Zhou et al. (2016), allohexaploids from *B. juncea* and *B. oleracea* which were used to study chromosome loss: from the findings, A and B genomes were retained over C genome chromosomes, which in some cases would be entirely lost in subsequent generations.

Doubling chromosomes in ABC triploids to produce AABBCC hexaploids by soaking the auxiliary meristem tissue with colchicine has previously been shown to be successful (Pradhan et al. 2010). Confirmed ABC 3*x* triploids from *B. oleracea* × *B. juncea* crosses were multiplied as cuttings and chromosome multiplication induced by soaking the auxiliary meristem in colchicine concentrations of 0%, 0.05%, 0.1%. 0.15%, 0.2% and 0.25% (w/v). Changes were seen in pods, leaves, and stems in all groups relative to the untreated controls, and all colchicine treatment categories produced seeds. Although the triploid hybrids were sterile, induction of allohexaploids through colchicine treatment restored fertility in these plants, with a total of 200 seeds harvested from this cross combination. Generally, hybrids generated from interspecific crosses with their wild relatives have low fertility and sterility, although doubling of chromosomes in some cases does seem to restore fertility (Yao et al. 2012), as was also seen in our study.

Seeds obtained from the cross *B. oleracea* × *B. juncea* (S_0 population) were sown and preliminary characterization carried out. Seed germination of putative allohexaploid S_1 plants was 70.5%, with a total of 94 plants from 140 seeds planted successfully germinating. Phenotypic assessment of the S_1 generation was indicative of hybrid vigour from the enlarged leaves and thick stems observed, with up to 84% pollen viability. Genotypic analysis of these S_1 allohexaploid *Brassica* is planned to unravel the degree of A/B/C chromosome interactions and determine genome stability in this newly developed allohexaploid. Furthermore, this new germplasm material comprises potential stock for future genetics studies and breeding experiments as well as diversification of the allohexaploid *Brassica* crop germplasm pool and can also be used as a bridge to transfer useful phenotypic traits into other cultivated *Brassica* crop species.

6. General discussion and future direction

In the face of challenges posed by climate change and the growing global population, there is an urgent need to increase food production and enhance agricultural systems (Batley and Edwards 2016). An enhanced agricultural system should be defined as a system in which high crop yield and quality can be obtained despite environmental stresses. A promising approach to provide this is offered by plant breeding and the release of tolerant varieties. The *Brassica* genus contains several economically important crop species with major contributions to human nutrition. Thus, combining these species to develop new allohexaploid *Brassica* crops could substantially benefit agricultural production. However, to date there are not many success stories related to the creation of a new allohexaploid *Brassica* species, due to challenges caused by aneuploidy, infertility and meiotic instability. This thesis contributes to the overall pool of knowledge on genetic stability and fertility and the genotypic variation involved in novel allohexaploid *Brassica* populations of different types, as well as producing brand new germplasm to broaden the genetic diversity of this developing crop type.

In the first study in this thesis, two second generation allohexaploid *Brassica* populations from the crosses *B. rapa* × *B. carinata* (A2 population) and (*B. napus* × *B. carinata*) × *B. juncea* (H2 population) were analysed for fertility and meiotic stability. From the results, genotype and progeny set in the heterozygous H2 population were both influencing fertility and stability. Heterosis, which is described as the presence of genetically superior traits in hybrids arising from crosses between different parents (Fu et al. 2015), was present in both allohexaploid populations from the increased plant heights in the progeny populations when compared to parents. The low fertility witnessed in the A2 and H2 populations is a common phenomenon in newly formed polyploids. In this study, the variable fertility observed may also be attributed to ongoing segregation for meiotic stability.

In the next study in this thesis, hypotheses relating to genetic non-identity of an unstable double haploid population were tested. I sought to determine if non-homologous translocations in microspore-derived allohexaploids would lead to instability and further non-homologous translocations in subsequent generations by comparing progeny within lines and between the first and second generations. While lines in two second generation progeny sets were identical to their parents, I found genetic-non-identity in four progeny sets. SNP genotyping analysis and Copy Number Variation plots revealed the occurrence of inherited as well as novel

genomic rearrangements in the second-generation lines. Segregation of non-homologous translocation segments was evident in one progeny set, and surprisingly appeared to be influencing fertility.

In the final study in this thesis, interspecific hybridisation and ovule culture were used to develop novel hybrids from *B. juncea* crossed with wild C genome species and *B. oleracea*. I characterized interspecific hybrids obtained using phenotypic analysis, pollen fertility analysis and flow cytometry. Production of hybrids between allotetraploid *Brassica juncea* (AABB) and wild C genome (CC) species (2n = 18) was unsuccessful. However, I developed and confirmed up to seven triploid 3*x* hybrids using *B. juncea* by *B. oleracea* interspecific crosses. Successful triploid 3*x* hybrids were treated with colchicine to induce chromosome doubling to create a putative allohexaploid *Brassica.* Drawing from this experience, I highlight challenges in developing interspecific crosses, arising mainly due to hybridisation barriers preventing successful fertilization, but also because wild species are biennial with long vegetative phases presenting challenges while making successful hybrids.

Following findings in this thesis, several studies may be conducted in future. Studies in *Brassica napus* have shown that the gene *PrBn* affects crossover frequency in AC allohaploids (Jenczewski et al. 2003), but no gene affecting the frequency of non-homologous recombination has so far been identified in *Brassica*, and the genomic factors controlling meiosis in allohexaploid *Brassica* remain largely unknown. However, in allohexaploid wheat, the *Ph1* locus is known to regulate meiotic interactions, thus enabling the A, B and D genomes to pair and segregate faithfully with their homologs (Griffiths et al. 2006; Gaeta and Pires 2010). Meanwhile, Oilseed rape (*B. napus* (AACC)) formed through ancestral hybridisation events between *B. rapa* (AA) and *B. oleracea* (BBCC) (U 1935) is a naturally stable allotetraploid species. However, synthetic *B. napus* and *Brassica* interspecific hybrids with the A and C genomes like the allohexaploids usually show unstable meiosis (Song et al. 1995; Tian et al. 2010; Szadkowski et al. 2011; Xiong et al. 2011; Zhou et al. 2016). Geng et al. (2013) reports hexaploid *Brassica* from various backgrounds as exhibiting instability in the early generations. However, Gupta et al. (2016) identified a *B. rapa* genotype that when crossed with different *B. carinata* accessions reliably conferred stable 27 bivalents at meiosis across several generations and locations. A question thus arises: are there genetic factors present in *B. rapa* or *B. oleracea* which confer meiotic stability? Mason and Batley (2015) suggest that *B. napus*

may have inherited the genetic control seen at meiosis either by mutation or through the accumulation of minor alleles from the diploid parents.

Future work to identify the genetic factors controlling stability in *Brassica* allohexaploids created from crosses between different species and genotypes is necessary. Production of mapping populations between unstable and stable lines, or association mapping of meiotic stability and fertility with genotypes in large populations produced from diverse germplasm would be helpful in elucidating these factors. Association mapping describes analysis of statistical associations between genetic markers, such as individual SNPs or SNP haplotypes, and phenotypic traits (Hayward et al. 2015). Association mapping is used in natural populations which have a rich genetic diversity to detect associations of DNA-based markers with traits useful in agriculture. Genetic mapping in polyploids can be challenging (Bevan et al. 2017), in *B. napus* ancient polyploidy events resulted in numerous duplicated segments and homoeologous regions, thus discriminating between two homologous sequences and two nearly-identical homoeologous sequences is complex (Huang et al. 2013). However, current advances in genomics and the availability of different technologies, each bearing different costs and efficiencies have led to an improvement in genomics-based strategies in plant breeding (Batley and Edwards 2016; Goodwin et al. 2016).

Other strategies such as bulked segregant analysis (BSA) may also be useful. When BSA is coupled to more recent next generation sequencing (NGS) technologies, it provides a fast and easy method for identifying molecular markers tightly linked to the causal gene/s underlying a given phenotype (Song et al. 2017). A segregating population from a genetic cross is developed, then the individuals are assayed for the focal trait and two pools (bulks) of segregants are created by selecting individuals from the tails of the phenotypic distribution (Magwene et al. 2011). BSA is a cost-effective method, as only genotyping the pooled DNA from individuals with similar phenotypes is required. It is also an efficient strategy for the detection of large effect QTL alleles in a large sample of progenies at a cheaper cost (Hu et al. 2012). Allelic variation can be compared, and comparison can be done where differences occur between the perceived stable progeny sets and unstable sets. Advancing the heterozygous H2 allohexaploid populations to later generations and characterization of this material to determine which genotypes and karyotypes confer increased meiotic stability and fertility may be illuminating. Further investigation of the microspore-derived lines identified to be segregating

for fertility may also shed light on the role of chromosome rearrangements in conferring fertility and meiotic stability in allohexaploids, as this material is 100% homozygous for parental alleles.

Polyploidy and interspecific hybridisation are often associated with benefits such as higher allelic diversity, increased vigour and colonization of a wider environmental niche (Udall and Wendell 2006). Additionally, genomic and genetic changes often accompany the successful merger of genomes with the genetic diversity generated often facilitating speciation and adaptation (Leitch and Leitch 2008). Allohexaploid *Brassica* is a largely understudied germplasm type, with much of the focus being in use of this material as a bridge to transfer traits of interest between the diploid and allotetraploid species rather than in creation of a new species (Chen et al. 2011). The new *B. oleracea* and *B. juncea* allohexaploid types that have been produced in this project could be of interest in determining changes in gene expression and epigenetic regulation resulting from polyploidy and hybridisation over several generations in homozygous material. The new allohexaploid population will be further characterized for fertility and genomic stability in the S_1 generation and beyond, which presently, is outside the scope of this thesis. Additionally, it may also be used as stock for future breeding and genetics experiments, particularly as a source of disease resistance, characterization of its nutritional profile, potential for or any potential for edible oil, animal feed and as raw material for production of renewable energy (biofuel).

Crop improvement efforts including genetic recombination consumes much time and is often a laborious exercise. Availability of genomic resources such as the 60K and 90K SNP Infinium Illumina genotyping arrays for *Brassica* will hopefully enable identification of genotypic and species-specific variability for meiotic stability in allohexaploid *Brassica* in future (Mason et al. 2014). High density SNP arrays continue to be successfully used in various studies in economically important crops and animals, examples include the 44K SNP array in rice, the 50K SNP array in maize and the 90K SNP array in wheat (Wang et al. 2014). Additionally, in future breeding strategies, researchers exploring wild or novel allohexaploid *Brassica* genes may exploit genome editing tools e.g. CRISPR (clustered regularly interspaced short palindromic repeat)/Cas 9 system. This gene editing strategy can be used to introduce desired target genes or group of genes within single generation, increasing genetic variation and achieving breeding goals much faster (Scheben et al. 2017). By using these genomic resources,

Brassica researchers and breeders can finally understand the complex homoeologous interactions between the different genomes within species with agronomic potential. Information generated is likely to provide further insight into genetic control and stability in new allohexaploids and how this can be exploited to further establish additional *Brassica* species for food and agricultural benefit.

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Appendix

Supplementary Table 1: Duplications and deletions in the MDL2 population derived from microspores of a (*Brassica napus* × *B. carinata*) × *B. juncea* allohexaploid hybrid: (0; absent, 1; homozygous (AA or BB), 2; duplication (AB), -; deletion, **; segregation)

Line	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	C1	C2	C3	C4	C5	C6	C7	C8	C9
MDL23	1	0	1	1	-	1	1	1	0	1	1	1	1	1	2	1	1	1	2
MDL23_01	1	0	1	1	-	1	1	1	0	1	1	1	1	1	2	1	1	1	2
MDL23_02	1	0	1	1	-	1	1	1	0	1	1	1	1	1	2	1	1	1	2
MDL23_03	1	0	1	1	-	1	1	1	0	1	1	1	1	1	2	1	1	1	2
MDL23_04	1	0	1	1	-	1	1	1	0	1	1	1	1	1	2	1	1	1	2
MDL23_05	1	0	1	1	-	1	1	1	0	1	1	1	1	1	2	1	1	1	2
MDL28	2	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_01	2	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_02	2	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_03	2	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_04	2	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1

MDL28_05	2	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_06	-	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_07	-	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_08	-	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_09	-	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL28_10	-	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
MDL60	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_01	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_02	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_03	1	0	2	1	1	1	1	1	1	2	0	1	0	2	1	1	1	1	1
MDL60_04	1	0	2	1	1	1	1	1	0	2	1	1	0	2	1	1	1	1	1
MDL60_05	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_06	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_07	1	0	**	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_08	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_09	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1
MDL60_10	1	0	2	1	1	1	1	1	1	2	1	1	0	2	1	1	1	1	1

MDL64	0	0	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
MDL64_01	0	0	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
MDL64_02	0	0	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
MDL64_03	0	0	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
MDL64_04	0	0	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
MDL7	1	0	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1
MDL7_01	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_02	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_03	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_04	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_05	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_06	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_07	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_08	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MDL7_09	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1